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(57) Abstract

This invention relates uses of components of plant-like metabolic pathways not including psbA or PPi phosphorfructokinase and not generally operative in animals or encoded by the plastid DNA, to develop compositions that interfere with Apicomplexan growth and survival. Components of the pathways include enzymes, transit peptides and nucleotide sequences encoding the enzymes and peptides, or promoters of these nucleotide sequences to which antibodies, antisense molecules and other inhibitors are directed. Diagnostic and therapeutic reagents and vaccines are developed based on the components and their inhibitors. A cDNA sequence that encodes chorismate synthase expressed at an early state of Apicomplexan development, is disclosed and may be altered to produce a "knockout" organism useful in vaccine production.

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ANTIMICROBIAL AGENTS, DIAGNOSTIC REAGENTS, AND VACCINES BASED ON UNIQUE APOCOMPLEXIAN PARASITE COMPONENTS

Inventors: **Rima L.W. McLeod *et al.***

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This invention relates uses of components of plant-like metabolic pathways not including psbA or PPi phosphorfructokinase and not generally operative in animals or encoded by the plastic DNA, to develop compositions that interfere with Apicomplexan growth and survival. Components of the pathways include enzymes, transit peptides and nucleotide sequences encoding the enzymes and peptides, or promoters of these nucleotide sequences to which antibodies, antisense molecules and other inhibitors are directed. Diagnostic and therapeutic reagents and vaccines are developed based on the components and their inhibitors. A cDNA sequence that encodes chorismate synthase expressed at an early state of Apicomplexan development, is disclosed and may be altered to produce a "knockout" organism useful in vaccine production.

BACKGROUND

Apicomplexan parasites cause the serious diseases malaria, toxoplasmosis, cryptosporidiosis, and eimeriosis. Malaria kills more than 2 million children each year. Toxoplasmosis is the major opportunistic brain infection in AIDS patients, causes loss of life, sight, hearing, cognitive and motor function in congenitally infected infants, and considerable morbidity and mortality in patients immunocompromised by cancer, transplantation, autoimmune disease and their attendant therapies. Cryptosporidiosis is

an untreatable cause of diarrhea in AIDS patients and a cause of epidemics of gastrointestinal disease in immunocompetent hosts. *Eimeria* infections of poultry lead to billions of dollars in losses to agricultural industries each year. Other Apicomplexan infections, such as babesiosis, also cause substantial morbidity and mortality. Although there are some methods for diagnosis and treatment of Apicomplexan caused diseases, some of these treatments are ineffective and often toxic to the subject being treated.

The tests available to diagnose Apicomplexan infections include assays which isolate the parasite, or utilize light, phase, or fluorescence microscopy, ELISAs, agglutination of parasites or parasite components to detect antibodies to parasites, or polymerase chain reaction (PCR) to detect a parasite gene. Most of the assays utilize whole organisms or extracts of whole organisms rather than recombinant proteins or purified parasite components. In many instances, the available assays have limited ability to differentiate whether an infection was acquired remotely or recently, and are limited in their capacity to diagnose infection at the outpatient or field setting.

The primary antimicrobial agents used to treat toxoplasmosis are pyrimethamine (a DHFR inhibitor) and sulfadiazine (a PABA antagonist). The use of pyrimethamine is limited by bone marrow toxicity which can be partially corrected by the concomitant administration of folinic acid. *T. gondii* cannot utilize folinic acid but mammalian cells can. Another problem is that pyrimethamine is potentially teratogenic in the first trimester of pregnancy. The use of sulfonamides is limited by allergy, gastrointestinal intolerance, kidney stone formation and Stevens-Johnson syndrome.

There are a small number of antimicrobial agents utilized less frequently to treat toxoplasmosis. These include clindamycin, spiramycin, azithromycin, clarithromycin

and atovaquone. Usefulness of these medicines for treatment of toxoplasmosis is limited by toxicities including allergy and antibiotic-associated diarrhea, (especially *Clostridium difficile* toxin associated colitis with clindamycin use). Lesser or uncertain efficacy of macrolides such as spiramycin, azithromycin, and clarithromycin also limits use of these antimicrobial agents. Atovaquone treatment of toxoplasmosis may be associated with lack of efficacy and/or recrudescent disease. There are no medicines known to eradicate the latent, bradyzoite stage of *T. gondii*, which is very important in the pathogenesis of toxoplasmosis in immunocompromised individuals or those with recurrent eye disease.

Medicines used to treat malaria include quinine sulfate, pyrimethamine, sulfadoxine, tetracycline, clindamycin, chloroquine, mefloquine, halofantrine, quinidine gluconate, quinidine dihydrochloride, quinine, primaquine and proguanil. Emergence of resistance to these medicines and treatment failures due to resistant parasites pose major problems in the care of patients with malaria. Toxicities of mefloquine include nausea, vomiting, diarrhea, dizziness, disturbed sense of balance, toxic psychosis and seizures. Melfoquine is teratogenic in animals. With halofantrene treatment, there is consistent, dose-related lengthening of the PR and Qt intervals in the electrocardiogram. Halofantrene has caused first degree heart block. It cannot be used for patients with cardiac conduction defects. Quinidine gluconate or dihydrochloride also can be hazardous. Parenteral quinine may lead to severe hypoglycemia. Primaquine can cause hemolytic anemia, especially in patients whose red blood cells are deficient in glucose 6-phosphate dehydrogenase. Unfortunately, there are no medicines known to be effective in the treatment of cryptosporidiosis

To more effectively treat Apicomplexan infections, there is an urgent need for discovery and development of new antimicrobial agents which are less toxic than those currently available, have novel modes of action to treat drug resistant parasites that have been selected by exposure to existing medicines, and which are effective against presently untreatable parasite life cycle stages (e.g., *Toxoplasma gondii* bradyzoites) and presently untreatable Apicomplexan parasites (e.g., *Cryptosporidium parvum*). Improved diagnostic reagents and vaccines to prevent these infections are also needed.

Information available on Apicomplexan parasites has not yet provided keys to solutions to health problems associated with the parasites. Analogies to other organisms could provide valuable insights into the operations of the parasite. There are reports of Apicomplexan parasites having plastids, as well as the nuclear encoded proteins, tubulin, calmodulin, PPi phosphofructokinase and enolase, which are reported to be similar in part to, or homologous with, counterparts in plant-like, lower life forms and higher plants. There are reports of a plastid genome and components of a protein synthetic system in a plastid-like organelle of Apicomplexans. *Plasmodium* and *T. gondii* plastid DNA sequences were reported to have homologies to algal plastid DNA sequences. The plastid membrane of *T. gondii* was reported to be composed of multiple membranes that appear morphologically similar to those of plant/algal chloroplasts, except for the presence of two additional membranes in the *T. gondii* plastid, suggesting that it may have been an ancient algal endosymbiont. Some of these Apicomplexan proteins such as tubulin, calmodulin and enolase with certain plant-like features also are found in animals, and therefore may appear in the host as well as the

parasite. A homologue to a gene, *psbA* encoding a plant protein important for photosynthesis, also was said to be present in Apicomplexans. .

Certain herbicides have been reported to inhibit the growth of Apicomplexans. The herbicides which affect growth of Apicomplexans are known to affect plant microtubules or a plant photosynthetic protein. In addition, a compound, salicylhydroxamic acid, (SHAM), had been found to inhibit *Plasmodium falciparum* (malaria) and *Babesia microti*.

Techniques of medicinal chemistry and rational drug design are developed sufficiently to optimize rational construction of medicines and their delivery to sites where Apicomplexan infections occur, but such strategies have not yet resulted in medicines effective against Apicomplexans. Rational development of antimicrobial agents has been based on modified or alternative substrate competition, product competition, change in enzyme secondary structure, and direct interference with enzyme transport, or active site. Antisense, ribozymes , catalytic antibodies, disruption of cellular processes using targeting sequences, and conjugation of cell molecules to toxic molecules are newly discovered strategies employed to interrupt cellular functions and can be utilized to rationally develop novel antimicrobial compounds, but they have not yet been utilized to design medicines effective against Apicomplexans. Large scale screening of available compounds with recombinant enzymes is used to identify potentially effective anti-microbial agents.

Reagents to diagnose Apicomplexan parasite infections have been developed targeting components of Apicomplexans or immune responses to the parasites, using ELISA, western blot, and PCR technologies, but improved diagnostic reagents,

especially those that establish duration of infection or that can be used in outpatient settings are needed to diagnose Apicomplexan infections. No vaccines to prevent Apicomplexan infections are available for humans and only a live vaccine prepared for prevention of toxoplasmosis in sheep is available for livestock.

To summarize, Apicomplexan parasites cause substantial morbidity and mortality, and treatments against the parasites are suboptimal or non-existent. Improved antimicrobial compounds that attack Apicomplexan parasites are needed. Because the diseases Apicomplexan parasites cause in some instances are due to recrudescence of latent parasites, an especially pressing clinical problem is that there are no effective antimicrobial agents effective for treatment of these latent parasite life cycle stages, especially in sequestered sites such as the brain or eye. New approaches and drug targets are required. Better *in vitro* and *in vivo* assays for candidate compounds are also needed. Better diagnostic and therapeutic methods, reagents and vaccines to prevent these infections are needed.

SUMMARY OF THE INVENTION

This invention relates uses of components of plant-like metabolic pathways (not usually associated with animals, not encoded in the plastid genome, and not including psbA or PPi phosphofructokinase) to develop compositions that interfere with Apicomplexan growth and survival. Components of the pathways include enzymes, transit peptides and nucleotide sequences encoding the enzymes and peptides, or promoters of these nucleotide sequences, to which antibodies, antisense molecules and other inhibitors are directed. Diagnostic and therapeutic reagents and vaccines are

developed based on the components and their inhibitors. Attenuation of live parasites through disruption of any of these components or the components themselves provide vaccines protective against Apicomplexans.

Transit peptides are used to identify other proteins and their organelle targeting sequences that enter and exit from unique Apicomplexan organelles. The identified components are potential for production of medicines, reagents and assays, and vaccines. The protein which includes the transit peptide is not necessarily an enzyme in a biochemical pathway.

The methods and compositions of the present invention arise from the inventors' discovery that metabolic pathways, and targeting signals similar to those found in plants and algae, especially, but not exclusively those encoded within the nucleus, are present in Apicomplexan parasites. These plant-like pathways in Apicomplexan parasites are targetable by inhibitors, as measured by determining whether the inhibitors, either singly or in combination, are effective in inhibiting or killing Apicomplexan parasites *in vitro* and/or *in vivo*.

The present invention includes new methods and compositions to treat, diagnose and prevent human and veterinary disease due to Apicomplexan infections. The invention is based on applications and manipulations of components of algal and higher plant-like metabolic pathways discovered in Apicomplexan parasites. "Plant-like" means that products of the pathways, enzymes and nucleotide sequences encoding enzymes in the pathways, are homologous or similar to products, enzymes and nucleotide sequences known in plants, wherein plants include algae. As used herein, "plant-like" excludes metabolic pathways generally operative in or identical to those in

animals and pathways involving psbA or phosphofructokinase and those encoded by the plastid genome. The limits of a "pathway" are defined as they are generally known to those of skill in the art. Methods to detect plant counterparts in Apicomplexan include: a) immunoassays using antibodies directed to products and enzymes known in plants, b) hybridization assays using nucleotide probes that hybridize to specific sequences in plants; c) determining homologies of Apicomplexan nucleotide or protein sequences with plant nucleotide or protein sequences; and/or d) substrate tests for specific enzymatic activity.

The "plant-like" pathways of the present invention are identified by:

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a) identification of metabolic pathways characteristic of plants but not generally present in animals;

b) identification and characterization of Apicomplexan enzymes, nucleic acids and transit sequences as components similar or homologous to those in a);

c) identification and development of compounds (inhibitors) which abrogate the effect of the components of the pathways *in vitro* and *in vivo*, singly or in a plurality, against one or more types of Apicomplexan parasites and in conjoint Apicomplexan, bacterial and fungal infections.

The identified pathways are then used for:

a) rational design or selection of compounds more active than the known compounds (inhibitors), with good absorption following oral administration, with appropriate tissue distribution and without toxicity or carcinogenicity;

b) testing of such rationally designed compounds alone and together for safety, efficacy and appropriate absorption and tissue distribution *in vitro* and *in vivo*;

c) development and testing of diagnostic reagents and assays;

d) development and testing of live attenuated and component based vaccines.

By locating new targets in Apicomplexan pathways, doors now are open for development of more effective antimicrobial agents to treat Apicomplexan parasites in humans and agricultural animals. In addition, enzymes in these plant-like pathways provide improved diagnostic tests for diseases caused by Apicomplexans. Vaccines against infectious diseases caused by Apicomplexan parasites are derived from the novel compositions of the invention.

A method for inhibiting an Apicomplexan parasite, includes selecting the metabolic pathway of the present invention and interfering with the operation of the pathway in the parasite. The Apicomplexan parasite is preferably selected from the group that includes *Toxoplasma*, *Plasmodium*, *Cryptosporidia*, *Eimeria*, *Babesia* and *Theileria*. The pathway may utilize a component encoded by an Apicomplexan nuclear gene.

Suitable metabolic pathways or components include

a) synthesis of heme from glutamate and tRNA glu by the plant-like, heme synthesis (5 carbon) pathway (hereinafter the "heme synthesis pathway");

b) synthesis of C4 acids (succinate) by the breakdown of lipids into fatty acids and then acetyl CoA, and their use in the glyoxylate cycle (hereinafter the "glyoxylate cycle");

c) synthesis of chorismate from phosphoenolpyruvate and erythrose 4 phosphate by the shikimate pathway (hereinafter the "shikimate pathway").

d) synthesis of tetrahydrofolate from chorismate by the shikimate pathway.

e) synthesis of ubiquinone from chorismate by the shikimate pathway;

f) electron transport through the alternative pathway with use of the alternative oxidase (hereinafter the "alternative oxidase pathway");

g) transport of proteins into or out of organelles through the use of transit sequences;

h) synthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophan) from chorismate by the shikimate pathway;

i) synthesis of the menaquinone, enterobactin and vitamin K1 from chorismate by the shikimate pathway;

j) synthesis of the branched chain amino acids (valine, leucine and isoleucine) from pyruvate and ketobutyrate by the plant-like branched chain amino acid synthesis pathway;

k) synthesis of the "essential" (i.e., not synthesized by animals) amino acids, histidine, threonine, lysine and methionine by the use of plant-like amino acid synthases;

l) synthesis of linoleneic and linoleic acid;

m) synthesis of amylose and amylopectin with starch synthases and Q (branching) enzymes and their degradation;

n) synthesis of auxin growth regulators from indoleacetic acid derived from chorismate;

o) synthesis of isoprenoids (diterpenes, 5 carbon units with some properties of lipids) such as giberellins and abscidic acid by the mevalonic acid to giberellin pathway.

The interfering compositions are selected from the group consisting of enzyme inhibitors including competitors; inhibitors and competitive or toxic analogues of substrates, transition state analogues, and products; antibodies to components of the pathways; toxin conjugated antibodies or components of the pathways; antisense molecules; and inhibitors of transit peptides in an enzyme. In particular, the interfering compositions include gabaculine, 3-NPA, SHAM, 8-OH-quinoline, NPMG. Interfering with the operation of the metabolic pathway is also accomplished by introducing a plurality of compositions to the pathway, wherein each of the compositions singly interferes with the operation of the metabolic pathway. In certain instances, the plurality of compositions inhibits the parasite to a degree greater than the sum of the compositions used singly, that is, exhibits a synergistic effect. Embodiments of a plurality of compositions include gabaculine and sulfadiazine; NPMG and sulfadiazine; SHAM and gabaculine; NPMG and pyrimethamine; NPMG and cycloguanil (which inhibits Apicomplexan DHFR [TS]), and other inhibitors and competitors of interrelated cascades of plant-like enzymes. Wherein the effect of inhibitors together is greater than the sum of the effects of each alone, the synergistic combination retards the selection of emergence of resistant organisms and is more effective than the individual components alone.

In various embodiments, the interfering composition acts on a latent bradyzoite form of the parasite, or multiple infecting Apicomplexan parasites simultaneously, or on conjoint infections with other pathogenic microorganisms which also utilize the plant-like metabolic pathway.

A method of determining the effectiveness of a composition in reducing the deleterious effects of an Apicomplexan in an animal, include: a) identifying a composition that inhibits growth or survival of an Apicomplexan parasite *in vitro* by interfering with a plant-like metabolic pathway and b) determining a concentration of the composition in an animal model that is non-toxic and effective in reducing the survival of the parasite in the animal host and/or the deleterious effects of the parasite in the animal.

Developing a lead compound that inhibits an Apicomplexan parasite is accomplished by a) identifying a plant-like metabolic pathway in an Apicomplexan parasite and b) identifying a composition that interferes with the operation of the pathway as a lead compound.

A composition which inhibits a specific life cycle stage of an Apicomplexan parasite by interfering with a plant-like metabolic pathway that utilizes a component encoded by a nuclear gene includes gabaculine; a composition including an enzyme in a metabolic pathway in an Apicomplexan parasite that is selectively operative in a life-cycle stage of the parasite includes the enzymes alternative oxidase, and UDP glucose starch glycosyl transferase. A composition comprising SHAM and 8-OH-quinoline inhibits the alternative oxidase in the latent bradyzoite form of an Apicomplexan parasite.

A method to identify a plant-like gene encoding a component of a plant-like metabolic pathway in an Apicomplexan parasite is a) obtaining a strain of *E. coli* that is deficient for a component of the metabolic pathway, said deficiency causing the strain to require supplemented media for growth, b) complementing the *E. coli* with a gene

or portion of the gene encoding a component of the metabolic pathway in the Apicomplexan parasite; and c) determining whether the complemented *E. coli* is able to grow in unsupplemented media, to identify the gene.

Another method for identifying a plant-like gene product of a metabolic pathway in an Apicomplexan parasite is a) contacting the parasite with a gene probe; and b) determining whether the probe has complexed with the parasite from which the identity of the gene product is inferred.

A method for identifying a plant-like gene product of a metabolic pathway in an Apicomplexan parasite also includes: a) cloning and sequencing the gene; and b) determining whether the gene is homologous to a plant gene which encodes a plant enzyme with the same function.

A method for identifying a plant-like gene product in a metabolic pathway in an Apicomplexan parasite is a) contacting the parasite or its enzyme with a substrate for the plant-like enzyme; b) measuring enzyme activity; c) determining whether the enzyme is operative; and d) inhibiting activity of the enzyme in vitro with an inhibitor.

Identifying a gene or gene product in an Apicomplexan parasite which possesses an organelle transit sequence which transports a protein, wherein the protein is not necessarily an enzyme in a metabolic pathway, but is identified because it has a characteristic organelle transit sequence is also within the scope of the invention.

The invention also relates to a diagnostic reagent for identifying the presence of an Apicomplexan parasite in a subject, where the subject includes a domestic or livestock animal or a human The reagent may include all or a portion of a component of the plant-like pathway, an antibody specific for an enzyme that is a component of a

plant-like metabolic pathway in the parasite, or all or part of a nucleotide sequence that hybridizes to a nucleic acid encoding a component of the pathway. A diagnostic assay that identifies the presence of an Apicomplexan parasite or specific life-cycle stage of the parasite may use the diagnostic reagents defined herein.

A diagnostic reagent for identifying the presence of an Apicomplexan parasite, includes an antibody specific for an enzyme that is part of a plant-like metabolic pathway.

A diagnostic assay for the presence of an Apicomplexan parasite in a biological sample includes: a) contacting the sample with an antibody selective for a product of a plant-like metabolic pathway that operates in an Apicomplexan parasite; and b) determining whether the antibody has complexed with the sample, from which the presence of the parasite is inferred. Alternatively, the assay is directed towards a nucleotide sequence. In both these cases, appropriate antibody or nucleotide sequences are selected to distinguish infections by different Apicomplexans.

An aspect of the invention is a vaccine for protecting livestock animals, domestic animals or a human against infection or adverse consequences of infection by an Apicomplexan parasite. The vaccine may be produced for an Apicomplexan parasite in which a gene encoding a component of a plant-like metabolic pathway in the parasite is manipulated, for example, deleted or modified. When the gene is deleted or modified in the live vaccine, the component of the pathway may be replaced by the presence of the product of an enzymatic reaction in tissue culture medium. The vaccine strain can then be cultivated *in vitro* to make the vaccine.

A vaccine for protecting animals against infection by an Apicomplexan parasite is based on an Apicomplexan parasite in which the parasite or a component of a metabolic pathway in the parasite is used.

The vaccine may use a component of the pathway that is operative at a particular life stage of the parasite. A suitable component is the *AroC* gene from *T. gondii* or *P. falciparum*.

A method of treatment for an infection in a subject by an Apicomplexan parasite includes the following steps: a) obtaining an inhibitor of a plant-like metabolic pathway in an Apicomplexan parasite; and b) administering an effective amount of the inhibitor to the subject.

BRIEF DESCRIPTIONS OF DRAWINGS

FIG. 1A-C illustrates the heme synthesis pathway and the effect of GSAT in *T. gondii*.

FIG. 1A diagrams the heme synthesis pathway. FIGS. 1B and 1C show that uptake of tritiated uracil by tachyzoites (RH strain) is inhibited by gabaculine, an inhibitor of GSA aminotransferase. P/S = pyrimethamine and sulfadiazine. Note that ALA synthase is also present in *T. gondii* and constitutes an alternative pathway for heme synthesis

FIG. 2A-B shows unique lipid degradation in the glyoxylate cycle in *T. gondii*.

FIG. 2A is a schematic representation of the glyoxylate cycle. FIG. 2B shows uptake of tritiated uracil by tachyzoites (RH strain) is inhibited by 3-NPA (0 005 to 5

mg: G/ML). Note this inhibitor also effects succinate dehydrogenase, so its inhibitory effect does not unequivocally support presence of the glyoxylate pathway.

FIG. 3A is a schematic representation of a pathway which demonstrates alternative oxidase as an alternative pathway for generation of energy in Apicomplexan parasites. FIG. 3B shows that uptake of tritiated uracil by tachyzoites (RH strain) is inhibited by SHAM.

FIG. 4A is a schematic representation of the pathway for conversion of shikimate to chorismate in *T. gondii.*. The inhibitor of EPSP synthase is NMPG. FIG. 4B shows uptake of tritiated uracil by tachyzoites (RH strain) is inhibited by NPMG. Toxicity of NPMG was assessed by its ability to prevent growth of human foreskin fibroblasts (HFF) after 4 days, as measured by tritiated thymidine uptake and microscopic evaluation. FIG. 4C shows product rescue of NPMG's inhibitory effect on EPSP synthase by PABA. The effect of PABA on sulfadiazine is similar, but the effect on pyrimethamine, as predicted reduces the enzyme to the levels that were present when media alone was utilized, as measured by the uracil uptake.

S	=	sulfadiazine
PYR	=	pyrimethamine
PABA	=	para amino benzoic acid.

FIG. 4D shows functional and enzymatic evidence for the shikimate pathway in *T. gondii* with inhibition of EPSP synthase enzyme activity by 1 mM glyosate. Squares, without glyphosate. Circles, with glyphosate FIG. 4E shows evidence for the shikimate pathway in *P. falciparum* with functional evidence for the shikimate pathway in *P. falciparum* Glyphosate inhibition of *in vitro* growth of asexual erythrocytic

forms and PABA and folate antagonism of growth inhibition. Effect of NPMG on *C. parvum* was not abrogated by PABA. This suggests that either uptake of PAPA by *C. parvum* differs or effect of NPMG is on a different branch from the shikimate pathway in *C. parvum*.

FIG. 5 is a schematic representation of interrelationships of metabolic pathways in Apicomplexan parasites.

FIG. 6 shows inhibitory effects of NPMG, gabaculine, SHAM 8-OH-quinoline on *Cryptosporidia*. 3NPA also inhibited *Cryptosporidia*.

FIG. 7 shows the effects of gabaculine (20 mM) on growth of tachyzoites/bradyzoites (R5) in human foreskin fibroplasts, over 8 days as determined by uracil uptake. Note increased uptake of uracil by the 8th day.

FIG. 8 shows the effect of NPMG, pyrimethamine, and pyrimethamine plus NPMG on survival of mice following intraperitoneal infection with 500 tachyzoites of the RH strain of *T. gondii*. Dosage of NPMG was 200mg/kg/day and pyrimethamine was 12.5 mg/kg/day).

FIG. 9 shows nucleotide and deduced amino acid sequences of *T. gondii* chorismate synthase cDNA. The asterisk indicates the stop codon.

FIG. 10 shows results of CLUSTAL X alignments of the deduced amino acid sequences if the putative *T. gondii*, chorismate synthase with the corresponding sequences from *Synechocystites, S. cerevisiae, S. lypocersicum, N. crassa* and *H. influenza*. Dashes were introduced maximize alignment. Amino acids which are identical in all 6 organisms are underlined. The percent identity of the chorismate synthase from each organism with the *T. gondii* protein was calculated to be as follows

Synechocystis (51.4%), *S. cerevisiae* (49.6%), *S. lycopersicum* (47.2%), *N. crassa* (45.0%) and *H. influenza* (44.5%). The large internal regions in the *T. gondii* sequence which have no counterparts in the chorismate synthases of other organisms, were not included in this calculation.

FIG. 11 shows the transit sequences of *Zea mays* and *T. gondii* chorismate synthases. The sequences of the transit peptide directing the transport of the wx+ protein into maize amyloplasts and chloroplasts and the portion of the *T. gondii* chorismate synthase sequence which is homologous are aligned. The amino acid sequence is given in one letter code. * indicates an identical amino acid in the *Wx Zea mays* and *T. gondii* sequences. • indicates homologous amino acids in the *Wx Zea mays* and *T. gondii sequences.*

The transit sequence in the *Wx Zea mays* protein (UDP-glucose-starch-glycosyl transferase) begins at amino acid number 1 and ends at amino acid number

72. The portion (amino acids 359 to 430) of *P. falciparum AroC* which corresponds to the novel internal sequence of the *T. gondii AroC* which includes the amino acids homologous to the maize protein, is as follows:

IPVENMSTKKESDLLYDDKGECKNMSYHSTIQNNEDQILNSTKGFMPPKNDKNFNNIDDYNVTFNNNEEKLL

The *T. gondii* portion of the *AroC* (chorismate synthase) sequence which demonstrates 30% homology begins at amino acid number 330 and ends at amino acid number 374. The first (single) arrow indicates the processing site of *Zeamays* UDP glucose Gylcosyl transferase transit peptide and the second (double) arrow indicates the location at which the mature protein begins.

FIG. 12 shows *P. falciparum*, chorismate synthase cDNA and deduced amino acid sequences.

FIG. 13 shows a genomic sequence of *T. gondii* chorismate synthase.

FIG. 14 shows (A) a *T. gondii* cDNA chorismate synthase DNA construct which is useful to produce antibody or a vaccine; (B) a Western blot (arrows mark chorismate synthase).

FIG. 15 shows green fluorescent (gfp) protein expression in a stably transfected tachyzoite; this tachyzoite has a reporter construct, a chorismate synthase-gfp; gfp is cytoplasmic (grey) and a defined structure in the area of the plastid is the white dot; the nucleus is the larger red area; gfp is in the cytoplasm.

FIG. 16 shows life cycle stage associated expression and localization of chorismate synthase in *T. gondii*.

(A) Tachyzoites: (1) 15 days - - Double stained with tachyzoite surface antigen 1 (SAG1) (perimeter raised, top and bottom left) and DNA stain (DAPI) (bottom right) and chorismate synthase (top right, white); (2) Double stained with dense granule protein 4 (granular stain, top left), chorismate synthase (white); p30, lower right panel, (perimeter raised) rhoptry probe (raised grey, rhop); (3) Double stained chorismate synthase-punctate white, SAG1 (P30, perimeter raised). (Note discrete punctate white area of chorismate synthase staining in perinuclear area, the customary subcellular location of the plastid).

(B) Bradyzoites: (1) Abbreviations are the same as in A; Note diffuse granular appearing cytoplasmic staining of bradyzoite chorismate synthase (top

right); (2) Immunoperoxidase stain with antibody to recombinant chorismate synthase shows diffuse cytoplasmic darker staining.

(C) Microgametes (Mi), Macrogametes (Ma); Note darker immunoperoxidase staining of these forms but not schizonts (s) in cat intestine.

(D) Chorismate synthase mRNA production in tachyzoites and with bradyzoites; Note SAG1 message for a tachyzoite protein, BAG 1-5 message for a bradyzoite protein and constitutively expressed mRNA for tubulin.

FIG. 17 shows: (a) schematic illustration of glyoxylate cycle, (b) inhibitors of isocitrate lyase (ICL), (c) *T. gondii* isocitrate lyase enzyme activity, (d) inhibition of ICL enzyme activity by 3NPA, and (e) inhibition of tachyzoites in tissue culture.

FIG. 18 shows a *T. gondii* isocitrate lyase (ICL) cDNA sequence.

FIG. 19 shows a *T. gondii* isocitrate lyase (ICL) amino acid sequence.

FIG. 20 shows (a) *T. gondii* isocitrate lyase (ICL) binding pocket and active site inside box, and (b) comparison with the published sequence of yeast isocitrate lyase with mutated lysine (K) which inactivated the enzyme (arrows).

FIG. 21 shows a *T. gondii* isocitrate lyase genomic DNA sequence (ICL).

FIG. 22 shows *T. gondii* isocitrate lyase in bradyzoites; Note brown areas in immunoperoxidase stain preparation.

FIG. 23 shows isocitrate lyase (a) in a western blot of tachyzoites (b) during stage conversion, and (c) mRNA during stage conversion. (Abbreviations are the same as in FIG. 16A and D legends).

FIG. 24 shows enzymatic, genetic, functional activity of Apicomplexan parasites and its inhibition and show *T. gondii* acetyl coA carboxylase is inhibited by -fop herbicides:

(A) Acetyl coA carboxylase enzyme activity is inhibited by -fop herbicides;

(B) *T. gondii* growth in tissue culture inhibited by compounds that inhibit acetyl coA carboxylases;

(Note the inhibitor activity is parallel to that in FIG. 24A. Clodinafop is a lead compound. *T. gondii* uptake of 3H uracil is inhibited by fop herbicides.)

(C) Effect of clodinafop on *T. gondii* with 4 days in culture then removal of the herbicide for 2 days. Note plaques (A) and (C) higher view of

replicating parasites in these plaque controls and complete eradication of parasites in clodinafop (10(M) treated cultures;

Related sequences of Apicomplexan acetyl coA carboxylases; sequences of acetyl coA carboxylase biotin carboxylase domains from apicomplexan parasites are as in Genbank Accession Numbers AF 157612-16. Also, a domain swap yeast with the *T. gondii* active site and recombinant enzymes made from a fragment of the *T. gondii* gene are amenable to high throughput screens;

Phylogeny of biotin carboxylase domains of apicomplexan accases;

Structures of herbicides that inhibit acetyl coA carboxylases.

DESCRIPTION OF THE PREFERRED EMBODIMENT

This invention uses components of plant-like interrelated metabolic pathways that are essential for growth or survival of Apicomplexan parasites. The pathways are generally not operative in animals and do not include psbA or PPi phosphofructokinase and are not encoded in the plastid. Components include enzymes, products, targetting peptides, nucleotide sequences encoding the enzymes or peptides, and promoters, as targets for specific inhibitors. Use of these pathways provide a rational and novel framework to discover, characterize and develop medicines, diagnostic reagents and vaccines for Apicomplexan parasites.

Medicines, diagnostic reagents and vaccines are based upon interrelated plant-like enzyme cascades involved in the synthesis or metabolism or catabolism of Apicomplexan nucleic acids, amino acids, proteins, carbohydrates or lipids, energy transfer and unique plant-like properties of these enzymes which are shared with, and provide a basis for, discovery of other parasite proteins which have unique organelle targeting signals or unique promoter regions of the genes which encode the proteins. Synergistic combinations of inhibitors of the enzymes or proteins or nucleic acids which encode them are particularly useful in medicines.

To select pathways for use in the invention:

a) plant textbooks and the published literature are reviewed for properties characteristic of plants, but generally not animals, databases such as Genbank or the

Apicomplexan ESTs are reviewed to identify homologous Apicomplexan and plant-like genes; and

b) Western, northern and southern analyses, PCR, and ELISAs are used to recognize, or are based upon, for example, plant proteins and genes, to determine whether components of the pathways are present in Apicomplexans;

c) cloning, isolation and sequencing of genes and creation of gene constructs are used to identify Apicomplexan plant-like genes and their functions;

d) assays of enzyme activity are used to determined the operation of plant-like systems;

e) functions of parasite enzymes or part of a parasite enzyme are demonstrated by complementation of a yeast or bacteria deficient in the enzyme, or product rescue, or other methods to demonstrate enzyme activity;

f) activity of compounds, (i.e., inhibitors) known to abrogate effect of the plant-like enzyme, protein, or nucleic acid which encodes them *in vitro* and *in vivo*, are tested singly or in a plurality, for ability to abrogate the enzyme activity and against Apicomplexan parasites alone or together, and in conjoint Apicomplexan, bacterial and fungal infections.

The general compositions of this invention are:

A. Inhibitory compounds based on:

a) targeting proteins by

(i) substrate competition and transition state analogues

(ii) product competition

(iii) alteration of active site directly or by modification of secondary structure or otherwise altering function of the active site

(iv) interfering with protein function with antibody

(v) targeting an organelle or protein within an organelle using a toxic compound linked to a targeting sequence.

b) targeting nucleic acids encoding proteins (antisense, ribozymes)

c) targeting a component of the protein or nucleic acid (as above)

B. Diagnostic reagents (genes, proteins, antibodies) in ELISAs, western blots, DNA, RNA assays

C. Vaccines (live knockout, live mutated, components - genes, proteins, peptides, parts of genes constructs, etc.)

Specific examples of components of plant-like Apicomplexan pathways are in Table 1. Compounds known to inhibit these enzymes or properties in Apicomplexans and/or other microorganisms are listed in Table 1, as are novel ways to target them in Apicomplexans.

Table 1A. Apicomplexan plant-like metabolic pathways, components and inhibitors

Function	Gene name	Enzyme or property	Known inhibitors of enzymes or property	Basis for novel inhibitor
HEME SYNTHESIS	*HemL*	glutamate-1-semialdehyde aminotransferase (GSAT)	3-amino-2,3-dihydrobenzoic acid (Gabaculine); 4-amino-5-hexynoic acid; 4-amino-5-fluoropentanoic acid; 4-amino-5-hexynoic acid (γ acetylenic GABA); 2-amino-3-butanoic acid (vinyl glycine); 2-amino-4-methoxy-trans-3-butanoic; 4-amino-5-fluoropentanoic acid	S,AS,R
	GltX	glutamyl-tRNA synthase	———	
	HemA	glutamyl-tRNA reductase	———	
SHIKIMATE PATHWAY				
Chorismate synthesis	*AroA*	3-enolpyruvylshikimate phosphate synthase (3-phosphoshikimate-1 carboxyvinyltransferase)	N-(phosphonomethyl) glycine (glyphosphate), sulfosate, EPSP synthase Inhibitors 4 and 5, hydroxymaonate inhibitors of EPSP synthase**	S,AS,R
	AroB	dehydroquinate synthase (5-dehydroquinate dyhdrolase)		
	AroC	chorismate synthase 5-enolpyruvylshikimate 3-phosphate phospholyase)	———	
	AroC-ts	*AroC* transit sequence		
	AroD	dehydroquinate dehydratase	———	
	AroE	shikimate dehydrogenase	———	
	AroF	3-deoxy-d-arabino-heptulosonate 7 phosphate synthase	———	
	AroG	chorismate mutase (7-phospho-2-dehydro-3-deoxy-arabino-heptulate aldolase)	———	
	AroH	3-deoxy-d-arabino-heptulosante 7 phosphate synthase	———	
	AroI	shikimate 3-phosphotransferase (shikimate kinase)	———	

Function	Gene name	Enzyme or property	Known inhibitors of enzymes or property	Basis for novel inhibitor
Ubiqinone synthesis	*UbiA*	4-hydroxybenzoate octaprenyltransferase	———	S,AS,R
	UbiB	3-oxtaprenyl-4-hydroxybenzoate carboxylyase	———	
	UbiC	chorismate synthase	———	
Tyrosine synthesis	*TyrA*	prephenate dehydrogenase	———	S,AS,R
	TyrB	aromatic acid aminotransferase (aromatic transaminase)	———	
	TyrC	cyclohexadienyl dehydrogenase	———	
Tryptophan synthesis	*TrpA*	tryptophan synthase alpha sub unit	———	S,AS,R
	TrpB	tryptophan synthase beta sub unit	———	
	TrpC	indole-3-glycerol phosphate synthase (anthranilateisome-rase) (indoleglycerol phosphate synthase)	———	
	TrpD	anthranilate phosphoribosyltransferase	———	
	TrpE	anthranilate synthase component I	———	
	TrpF	phosphoribosyl anthranilate isomerase	———	
	TrpG	anthranilate synthase component II	———	
Phenylalinine Synthesis	*PheA*	Prephenate dehydratase (phenol 2-mono-oxygenase), chorismate mutase	———	S,AS,R
	PheB	Catechol 1,2-deoxygenase (phenol hydroxylase)	———	
	PheC	Cyclohexadienyl dehydrataseU	———	
Folate Synthesis	*pabA*	4-amino-4-deoxy chorismate synthase II, amidotransferase	———	S,AS,R
	pabB	4-amino-4-deoxy chorismate synthase I, binding component	———	
	pabC	4-amino-4-deoxy chorismate lyase	———	

Function	Gene name	Enzyme or property	Known inhibitors of enzymes or property	Basis for novel inhibitor
Menaquinone, enterobactin synthesis	*Enta*	Isochorismate synthase	———	S,AS,R
	Entb	2,3 dihydro 2,3 dihydroxy benzoate dehydrogenase	———	
	Entc	2,3 dihydro 2,3 dihydroxy benzoate synthetase	———	
ORGANELLE TRANSIT	*AroC-ts*	Transport into plastid, organelle targeting	———	S,AS,R
ALTERNATIVE RESPIRATION	*AOX*	Alternative oxidase	8-hydroxyquinoline, 3-hydroxyquinone, salicylhydroxamic acid, monoctone, benzhydroaxamic acid, m-Chlorohydroxamic acid, propylgallate, disulfuram, and others	S,AS,R,D
GLYOXYLATE CYCLE	*MS*	Malate synthase	———	S,AS,R
	ICL	Isocitrate lyase	3NPA, itaconic acid, 3 nitro propanol	

Key: S, modified substrate competitor; AS, antisense; R, ribozyme; Directed at active site, D; None known,

*EPSP synthase inhibitor 4 refers to 3-(phosphonooxy)-4-hydroxy-5-[N-(phosphonomethyl-2-oxoethyl)amino-1-cyclohexene-1-carboxylic acid (3α, 4α, 5β), compound with diethyl ethanamide EPSP synthase inhibitor 5 refers to shortened R phosphonate.

**A new, aromatic analogue of the EPSP synthase enzyme reaction intermediate 1 has been identified, which contains a 3- hydroxymalonate moiety in place of the usual 3-phosphate group. This simplified inhibitor was readily prepared in five steps from ethyl 3,4-dihydroxybenzoate. The resulting tetrahedral intermediate mimic is an effective, competitive inhibitor versus S3P with an apparent K(i) of 0.57 +/- 0.06 muM. This result demonstrates that 3- hydroxymalonates exhibit potencies comparable to aromatic inhibitors containing the previously identified 3-malonate ether replacements and can thus function as suitable 3-phosphate mimics in this system. These new compounds provide another example in which a simple benzene ring can be used effectively in place of the more complex shikimate ring in the design of EPSP synthase inhibitors. Furthermore, the greater potency of the tetraheral intermediate mimic versus the glycolate derivative and the 5- deoxy analog, again confirms the requirement for multiple anionic charges at the dihydroxybenzoate 5-position in order to attain effective inhibition of this enzyme.

The following were identified: inhibition of *Toxoplasma gondii* (*Tg*), *Plasmodium falciparum* (*Pf*), and *Cryptosporidium parvum* (*Cp*) EPSP synthase by N-phosphonomethylglycine (NPMG); *Tg* and *Pf* chorismate synthase (*AroC*) cDNA and deduced amino acid sequences; a novel sequence in the *Tg* chorismate synthase gene (*AroC-ts*) a portion of which is homologous with the plastid transit sequence of *Zea mays* (sweet corn) The *Pf* chorismate synthase (*AroC*) also has a corresponding novel and unique internal region *Cp, Eimeria bovis (Eb)* genomic DNA which hybridizes with *Tg AroC* (chorismate synthase) Inhibition of *Tg in vitro* by NPMG abrogated by para-aminobenzoate (PABA) Inhibition of *Pf in vitro* by NPMG abrogated by PABA and folate Inhibition of *Tg* EPSP synthase activity by NPMG *in vitro* Synergism of NPMG with pyrimethamine, with sulfadiazine and with SHAM

for *Tg in vitro*; Synergy of NPMG with pyrimethamine against *Tg in vivo*; SHAM and 8-hydroxyquinoline inhibited *Tg, Pf, Cp in vitro*; reactivity of *Tg* protein of ~66Kd with 5 antibodies (monoclonal and polyclonal to *VooDoo lily* and *T. brucei* alternative oxidases) and reduction to monomer similar to *VooDoo lily* and *T. brucei* alternative oxidases on a reducing gel; Identification of *Tg* cDNA and genomic DNA PCR products using primers based on conserved sequences in other alternative oxidases which are probed and sequenced; *Tg, Pf, Cp* inhibited by high concentrations of gabaculine. Reactivity of *Tg* protein of ~40Kd with 3 antibodies to GSAT (polyclonal α soybean, barley and synechococcus GSATs and not preimmune sera). Reactivity of *Cp* protein of ~40Kd with α barley GSAT. Inhibition of *Tg, Pf, Cp in vitro* by 3NPA; Reactivity of *Tg* protein with polyclonal antibodies to cotton malate synthase and cotton isocitrate lyase but not preimmune sera. In screening *Tg* cDNA library α GSAT antibody reactive clones are identified and are sequenced. *Tg* chorismate synthase and dehydroquinase enzymatic activities are demonstrated.

Table 1B. Components of Plant-Like Metabolic Pathways and Inhibitors

Function	Gene name	Enzyme or property	Known inhibitors of enzyme or property	Basis for novel inhibitor
BRANCHED-CHAIN AMINO ACID SYNTHESIS (VALINE, LEUCINE, ISOLEUCINE)	*ahas*	acetyhydroxy acid synthase	Imidazolinones imazquin=2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-quinolinecarboxylic acid; imazethapyr=2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-pyridinecarboxylic acid; imazapyr=()-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-pyridinecarboxylic acid, Sulfonylureas chlorimuron=2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoic acid; chlorsulfuron=2-chloro-N-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) amino]carbonyl] benzene sulfonamide; nicosulfurn=2-[[[[(4,6-dimethoxy-2-pyrimidinyl) amino] carbonyl] amino]sulfonyl]-N,N-dimethyl-3-pyridinecarboxamide; primisulfuron=2-[[[[(4,6-bis(difluoromethoxy)-2-pyrimidinyl) amino] carbonyl] amino]sulfonyl]benzoic acid; thifensulfuron=3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) amino] carbonyl]amino] sulfonyl]-2-thiophene-carboxylic acid; tribenuron=2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)methylamino]carbonyl]amino]sulfonyl]benzoic acid; sulfometuron=2-[[[[(4,6-dimethyl-2-pyrimidinyl) amino] carbonyl] amino]sulfonyl]benzoic acid; metsulfuron=2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl] benzoic acid, halosulfuron=, Sulfonanilides flumetsulam=N-(2,6-difluorophenyl)-5-methyl[1,2,4]triazolo[1,5-a]pyrimidine-2-sulfonamide	S,AS,R
	Kar	Keto-acid reducto isomerase	HOE 704	
	ipd	isopropylmalate dehydrogenase	0-isobutenyl oxalhydroxamate	

Function	Gene name	Enzyme or property	Known inhibitors of enzyme or property	Basis for novel inhibitor
SYNTHESIS OF ADDITIONAL "ESSENTIAL" AMINO ACIDS (e.g. histidine, methionine, lysine, threonine)				S,A,R,D
Histidine synthesis	*gpd+*	glycerol phosphate dehydratase	phosphon c acid derivatives of 1,2,4 triazole	
methionine synthesis	*ms+*	methionine synthesis+	———	
lysine synthesis	*ls+*	lysine synthesis+	inhibitors of lysine synthesis+	
Threonine synthesis	*ts+*	threonine synthesis+	———	
GLUTAMINE GLUTAMATE SYNTHESIS	*gs+*	glutamine synthase,	glufosinate=2-amino-4- hydroxy methyl phosphinyl, butaonic acid	S,AS,R,D
	gts+	glutamate synthetase*	———	
LIPID SYNTHESIS	*acc+*	acetyl co A carboxylase	Arloxyphenoxypro-pionates fenoxaprop=()-2-[4-[(6-chloro-2-benzoxazolyl)oxy] phenoxy]propanoic acid; fluazifop-P=(R)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid; quizalofop=()-2-[4-[(6-chloro-2-quinoxalinyl)oxy]phenoxy] propanoic acid, Cyclohexanediones clethodim=(E,E)-()-2-[1-[[(3-chloro-2-propenyl)oxy]imino] propyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclchexen-1-one; sethoxydim=2-[1-(ethoxyimino) butyl]-5-[2-(ethylthio)propyl]3-hydroxy-2-cyclohexen-1-one	S,AS,R,D
	ps	palmitic synthase		
	oas	oleic acid synthase		
	las	linoleic acid synthase		
	licas	linoleneic acid synthase		

Function	Gene name	Enzyme or property	Known inhibitors of enzyme or property	Basis for novel inhibitor
STARCH SYNTHESIS	*wx, gbss, sss*	UDP glucose starch glucosyl transferase (a starch synthase) other starch synthases	———	S,AS,R
	be, glgB, lgc, sbeI, II, III	Q or branching enzyme	———	
AUXIN GROWTH REGULATORS	———	Auxin analogue	**Phenoxyaliphatic acid** (2,4-D=(2,4-dichlorophenoxy) acetic acid; 2,4-DB=4-(2,4-dichlorophenoxy) butanoic acid; MCPP=; MCPA=(4-chloro-2-methylphenoxy) acetic acid; 2,4-DP=) **Benzoic acids** dicamba=3,6-dichloro-2-methoxybenzoic acid, **Picolinic acids (Pyridines)** picloram=4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; clopyralid=3,6-dichloro-2-pyridinecarboxylic acid; triclopyr=[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid; fluroxypry=[(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl) oxy]acetic acid;	S,AS,R
	ias	indoleacetic acid synthase	———	
GIBBERELLIN SYNTHESIS	*coaps*	copalylpyrophosphate synthase	Phosphon D, Amo-1618	S,AS,R
	ks	kaurene synthase	Cycocel	
	kox	kaurene oxidase	Phosphon D,	
	kaox	kaurene acid oxidase	Ancymidol, Paclobutrazol	
	gas	giberellic acid synthase	———	

Key S, modified substrate competitor; AS, antisense; R, ribozyme; D, direct inhibitor, alteration of target. These are suitable because they are unique to Apicomplexans. Unique to Apicomplexans means that either they do not exist in animals (e.g., acetohydroxyacid synthase, linoleic acid synthase, starch-amylose or amylopectin synthase, Q or branching enzyme, UDP glucose, starch glycosyl transferase or have unique antigenic or biochemical properties distinct from those of animals (e.g. acetylco A carboxylase)

*Also present in animals

•Other enzymes in these pathways unique to Apicomplexans

• Enzymes involved in the synthesis of these essential amino acids include the following:

Lysine: homocitrate synthase, homocitrate dehydrase (*Euglena, fungi*); aspartokinase, aspartate semialdehyde dehydrogenase, dihydropicolinate synthase, dihydropicolinate reductase, Δ^1 piperideine - 2, 6 - dicarboxylate transferase, N - succinyl - ε-keto- α aminopimelate transaminase, N - succinyl - L, L, α - ε-diaminopimelate desuccinylase, L, L α - ε diaminopimelate epimerase, meso-α ε diaminopimelate decarboxylase.
Inhibitors of lysine synthesis include: +2-4-Amino-4-carboxybutyl azridine-2-carboxylic acid(3) (aziridino-diaminopimelate [DAP], aziDAP); N-Hydroxy.DAP4; N-amino DAP5; 4 methyelene DAP 6, 3,4 didehydro DAP; 4 methylene DAP 4.
Methionine: L-homoserine acyltransferase, o-succinylhomoserine sulfhydrolase, L-homocysteine transferase, (to activate methionine - but not exclusively in plants: S-adenosylmethonine [SAM] synthase, SAM-methyltransferase, SAM decarboxylase, S-adenosylhomocysteine hydrolase)
Threonine: L homoserine kinase, O-phospho-L-homoserine (threonine) synthase
Isoleucine, valine: L-threonine deaminase, acetohydroxy acid synthase, acetohydroxy acid isomeroreductase, dihydroxy acid dehydrase, branched-chain amino acid glutamate transaminase.
Leucine: isopropylmalate synthase, α-isopropylmalate isomerase, β-isopropylmalate dehydrogenase, α ketoisocaproate transaminase.
Histidine: phosphoribulosyl formimino-5-aminolmidazol-4-carboxamide ribotide amidocyclase, imidazol glycerol phosphate dehydrase, imidazole acetol phosphate transaminase, histidinol phosphate phosphatase, L-histidinol dehydrogenase.

Additional herbicides which disrupt cell membranes include Diphenyl ethers [nitro phenyl ethers=] (acifluorfen=5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid;. fomeasafen=5-[2-chloro-4-(trifluoromethyl)phenoxy]-N-(methylsulfonyl)-2-nitrobenzamide; lactofen=()-2-ethoxy-1-methyl-2-oxoethyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate; oxyflurfen=2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene). Other bentazon=3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide above. Additional herbicides which disrupt pigment production include clomazone=2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone; amitrole=1H-1,2,4-triazol-3-amine; norflurazon=4-chloro-5-(methyl amino)-2-(3-(trifluoromethyl) phenyl)-3(2H)-pyridazinone; fluridone=1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone.

Enzymes in the heme synthesis [with a default ALA synthase pathway], shikimate pathway, alternative generation of energy and glyoxylate cycle are exemplified (Table 1A) and the others (Table 1B) are suitable for the practice of the invention.

As outlined succinctly above, the present invention includes new methods and compositions to treat, diagnose and prevent human and veterinary disease due to Apicomplexan parasites. Apicomplexan infections include those due to *Toxoplasma gondii* (toxoplasmosis), *Plasmodia* (malaria), *Cryptosporidia* (cryptosporidiosis), *Eimeria* (eimeriosis), *Babesia* (babesiosis), *Theileria* (theileriosis), *Neospora caninum*, and others. An Apicomplexan parasite, *Toxoplasma gondii*, is a representative of other Apicomplexan parasites because Apicomplexan parasites appear to be phylogenetically

related and have organelles and enzymes which are critical for their growth and survival. The presence of plant-like pathways/enzymes is confirmed in Apicomplexans by a) the effect of known inhibitors of the pathways in plants using *in vitro* and *in vivo* assays; b) Western, Northern and Southern hybridization analyses; c) isolation and comparison of relevant genes; d) demonstration of enzymatic activity; e) demonstration of immunologically reactive proteins which cross-react with proteins in plants; f) complementation of organisms which lack a gene or part of the gene encoding an enzyme with a parasite gene which encodes the enzyme; and/or g) recognition of plant-like transit sequences. *In vitro* assays include product rescue (*i.e.*, complete or partial abrogation of the effect of an inhibitor by providing the product of the reaction and thus bypassing the need for the enzyme which catalyzes the reaction. The assays are based on inhibition of the parasite *i.e.* restriction of growth, multiplication or survival of the parasite. Another measure of infection is "parasite burden" which refers to the amount (number) of parasites present as measured *in vivo* in tissues of an infected host. Another measure of infection is destruction of host tissues by the parasites. Inhibitors reduce parasite burden and destruction of host tissues caused by the parasites. Preferably the inhibitors must not be toxic or carcinogenic to the parasites' host and for *in vitro* assays not be toxic to cells in culture.

Enzymes of the newly detected plant-like pathways provide novel, unique and useful targets for antimicrobial therapy. These unique pathways and enzymes are within the plastid, glyoxosomes, cytoplasm or mitochondria. In addition, not suggested before for these parasites, some enzymes used in these pathways are encoded by genes within the nucleus

Plant-like pathways detected in Apicomplexan parasites include a) the 5-carbon heme biosynthesis pathway that utilizes glutamate as a carbon skeleton for synthesis and requires the unique enzyme glutamate-1-semialdehyde aminotransferase; b) the mobilization of lipids in the glyoxylate cycle which is a unique pathway that includes the enzymes isocitrate lyase and malate synthase; c) the generation of energy by an alternative pathway which includes a unique alternative oxidase and/or other unique pathways and enzymes for generating energy in the mitochondria or plastid; and, d) the conversion of shikimate to chorismate utilized in the synthesis of ubiquinone, aromatic amino acids and folate by plants, but not humans. The shikimate pathway includes the enzyme 3-phospho-5-enolpyruvylshikimate (EPSP) synthase, chorismate synthase, and chorismate lyase, as well as a number of enzymes unique to plants, fungi, bacteria, and mycobacteria, but not to animals. Inhibitors of some of these enzymes also provide information about the functioning and targeting of the enzymes.

The heme synthesis pathway involves enzymes encoded in the nucleus and imported to the plastid. This pathway is present in Apicomplexans including *T. gondii*, *P. falciparum*, and *Cryptosporidia parvum*. Inhibitors of the enzyme GSAT in the pathway include gabaculine (3-amino-2,3-dihydro benzoic acid), 4-amino-5-hexanoic acid, and 4-amino-5-fluropentanoic acid.

The glyoxylate cycle, reported to be present in plants, fungi, and algae, is also present in *T. gondii*. The cycle uses lipids and converts them to C4 acids through a series of biochemical reactions. One of the last steps in this series of reactions is dependent on the isocitrate lyase enzyme and another on the malate synthase enzymes Inhibitors of these enzymes include 3-nitropropionic acid and itaconic acid

The alternative respiratory pathway, present in a range of organisms including some bacteria, plants, algae and certain protozoans (trypanosomes), is present in *T. gondii*, *Cryptosporidia parvum*, and *Plasmodium falciparum* (in the latter parasite, two clones designated W2 and D6 were inhibited). The pathway is inhibited by a range of compounds including salicylhydroxamic acid, 8-hydroxyquinoline, Benzyhydroxamic acid (BHAM), m-Chlorohydroxamic acid (m-CLAM), Propylgallate, Disulfuram and others.

Enzymes involved in the synthesis of chorismate, including those which convert shikimate to chorismate, and enzymes which generate folate, aromatic amino acids and ubiquinone from chorismate in plants, are present in *T. gondii*, *Plasmodium falciparum*, *Cryptosporidium parvum*, and *Eimeria*. Inhibitors include N-(phosphonomethyl) glycine (glyphosate, sulfosate and others). A full-length *T. gondii* cDNA sequence encoding a chorismate synthase from this pathway and the deduced amino acid sequence provide information useful in developing novel antimicrobial agents. The *T. gondii* chorismate synthase has features in common with other chorismate synthases and entirely unique features as well. The unique features are novel sequences not shared with chorismate synthases from other organisms but with homology to an amyloplast/chloroplast transit sequence of *Zea mays* (sweet corn). A *P. falciparum* cDNA sequence encoding chorismate synthase and its deduced amino acid sequence also provide information useful for developing novel antimicrobial agents.

The genomic sequences provide information about regulation of the gene (e.g., unique promoter regions) and such unique regions enable targeting their regulatory elements with antisense.

A part of the novel internal sequence (i.e., SCSFSESAASTIKHERDGSAATLSRE RASDGRTTSRHEEEVERG) in the *T. gondii AroC* (chorismate synthase) gene has homology with the chloroplast/amyloplast targeting sequence of *Zea mays* (sweet corn) *wx* (UDP. glucose-starch-glycosyl transferase) protein (i.e., MAALATSQLVATRAGLGVPDASTFRRG AAQGLRGARASAAADTLSHRTSARAAPRHQQQARRGGRFPSLVVC). This transit sequence provides a novel way to target *T. gondii* enzymes that move from the cytoplasm into the plastid and is generally applicable to targeting any subcellular organelle. The *P. falciparum AroC* (chorismate synthase) has a corresponding novel internal sequence.

Additional pathways found in Apicomplexan parasites include the synthesis of branched chain amino acids (valine, leucine and isoleucine) and acetohydroxy acid synthase is the first enzyme in the branched chain amino acid synthesis pathway, inhibited by sulfonylureas and imidazolinones, as well as the synthesis of other "essential" amino acids, such as histidine, methionine, lysine and threonine. Starch synthesis, including starch synthases, the UDP-glucose-starch glycosyl transferase, and debranching enzymes and enzymes of lipid, terpene, giberellin and auxin synthesis, are part of other pathways in Apicomplexan parasites. Down modulation of the UDP-glucose starch glycosyl transferase pathway leads to a switch from amylose to amylopectin synthesis and thus the bradyzoite phenotype.

Demonstration of presence of one enzyme or the gene that encodes it in a known pathway implies presence of the full pathway. Thus, enzymes in parasite metabolic pathways that can be inhibited include: glutamyl-tRNA synthetase; glutamyl-tRNA reductase; prephenate dehydrogenase; aromatic acid aminotransferase (aromatic transaminase), cyclohexadienyl dehydrogenase; tryptophan synthase alpha subunit;

tryptophan synthase beta subunit; indole-3-glycerol phosphate synthase (anthranilate isomerase), (indoleglycerol phosphate synthase); anthranilate phosphoribosyltransferase; anthranilate synthase component I; phosphoribosyl anthranilate isomerase; anthranilate synthase component II; prephenate dehydratase (phenol 2-monooxygenase); catechol 1,2-deoxygenase (phenol hydroxylase); cyclohexadienyl dehydratase; 4-hydroxybenzoate octaprenyltransferase; 3-oxtaprenyl-4-hydroxybenzoate carboxylyase; dehydroquinate synthase (5-dehydroquinate hydrolase); chorismate synthase (5-enolpyruvylshikimate 3-phosphate phosph-lyase); dehydroquinate dehydratase; shikimate dehydrogenase; 3-deoxy-d-arabino-heptuloonate 7 phosphate synthase; chorismate mutase (7-phospho-2-dehydro-3-deoxy-arabino-heptulate aldolase); 3-deoxy-d-arabino-heptuloonate 7 phosphate synthase; shikimate 3-phosphotransferase (shikimate kinase); UDP glucose starch glycosyl transferase; Q enzymes; acetohydroxy acid synthase; glutamate-1-semialdehyde 2,1-aminotransferase; chorismate lyase; malate synthase; isocitrate lyase, and 3-enolpyruvylshikimate phosphate synthase (3-phosphoshikimate-1 carboxyvinyltransferase).

Recombinant protein produced by constructs with genes encoding these enzymes in *E coli* or in other expression systems is useful for producing antibodies and obtaining a crystal structure. Native enzyme is isolated. The expressed and native proteins are used to design and test new inhibitors in enzyme assays. Expressed and native (from varied life-cycle stages) proteins are used and the expressed protein is a source of the enzyme, and the enzyme assay is carried out in the presence and absence of the inhibitors, either alone or in combination and controls include the buffer for the

enzyme alone. The crystal structure is useful for characterizations of enzyme active site(s), secondary structure, transit sequence, substrate and product interactions. The design of additional inhibitors is carried out using published methods such as modifying substrates as had been done with inhibitors of EPSP synthase as well as high through put screening of available compounds.

Certain pathways are shown to be affected by inhibitors which are synergistic *in vitro*. Examples of synergistic inhibitors *in vitro* are gabaculine (heme synthesis) and SHAM (alternative energy generation); NPMG and SHAM; NPMG and sulfadiazine; and NPMG and pyrimethamine. Gabaculine and sulfadiazine are an additive combination *in vitro*.

An aspect of the invention is identifying potential targets for therapeutic intervention by considering nuclear as well as organellar genes as part of the production of enzymes for unique plant-like pathways. For example, the protein synthesis of plant-like proteins that is also demonstrated in Apicomplexan parasites suggests not only conservation of plastid genes but also conservation of nuclear genes which encode enzymes that act inside or outside the plastid, from an ancestor that is common to Apicomplexan parasites and algae. Many vital metabolic pathways of algae (often shared with their evolutionary relatives, higher plants) also are conserved in the Apicomplexan parasites, whether or not the pathways involve the plastid. Consequently, Apicomplexan parasites are sensitive to inhibitors that block several of these unique pathways. Combined attack on multiple targets retards the emergence/selection of resistant organisms. Considering nuclear and organellar genes has the dual advantage of rapidly identifying conservation of specific pathways and

simultaneously identifying both target sites and lead compounds for therapeutic drug development.

An aspect of the invention is a plurality of inhibitors, singly or in combination, directed against enzymes and/or genes encoding a different metabolic pathway. Examples of inhibitors suitable for practice of the present invention include GSAT, 3NPA, SHAM, 8-0H-quinoline, and NPMG, sulfonylureas, imidazolinones, other inhibitors of EPSP synthase or chorismate synthase which include competitive substrate analogues, transitional state inhibitors and direct active site inhibitors as well as other known compounds (Table I). Some pluralities of inhibitors produce synergistic effects.

Improved treatments against Apicomplexan parasites result from a variety of options:

1. some compositions may inhibit the operation of more than one pathway, thereby producing a strong effect and lessening the probability of resistance to the drug emerging because more than one mutation may be required;

2. some compositions may inhibit more than one step in a pathway;

3. some pluralities of compositions may have synergistic effects, producing more effective drugs; and

4. some compositions may target pathways operative exclusively during a life cycle of the parasite, making them more selective *e.g.* against the latent phase.

5. some compositions may inhibit other microorganisms (including other Apicomplexans.)

An additional detail of the invention is that representative Apicomplexan parasites, notably *T. gondii*, are used for assaying candidate inhibitors. The invention is

directed at effects of inhibitors of the unique plant-like pathways in Apicomplexan, alone and in combination. Organisms used for the assays include *T. gondii* tachyzoites, bradyzoites and a mutant that expresses 50% tachyzoite and 50% bradyzoite antigens. Unique plant enzymes and pathways that were found to be inhibited by compounds shown to inhibit plant pathways in Apicomplexans include: (1) glutamate-1 semialdehyde amino transferase, an enzyme important in heme synthesis, (2) isocitrate lyase, an enzyme important in utilization of lipids, (3) alternative oxidase enzyme complex; enzymes important in energy production and (4) 3-phospho-5-enolpyruvylshikimate synthase (EPSP synthase), an enzyme important in conversion of shikimate to chorismate which is a precursor for synthesis of folate, ubiquinone, and certain amino acids essential for survival.

The invention provides a rational, conceptual basis for development of novel classes of antimicrobial agents that inhibit Apicomplexan parasites, unique diagnostic reagents, and attenuated vaccines. The inhibitors provide lead compounds for the development of antimicrobial agents. Conserved enzyme active sites or parts of the molecules or genes that encode the protein which are targeted by the inhibitors provide the basis for development of new but related ways to target the enzymes, such as related protein inhibitors, intracellular antibodies, antisense DNA, and ribozymes.

Inhibitors are effective against more than one parasite (*e.g. T. gondii, P. falciparum* and *C. parvum*) and enzymes in these pathways also are present in other bacterial and fungal pathogens such as *Pneumocystis carinii, Mycobacterium tuberculosism Staphylococcus aureus*, and *Hemophilus influenza*, but not animals Thus, inhibitors of these pathways affect susceptible microorganisms which

concurrently infect a host. Because enzymes are utilized differentially in different parasite life-cycle stages, stage-specific inhibitors are within the scope of the invention. Genes encoding the enzymes in Apicomplexans are identifiable. The genes encoding the enzymes are effectively knocked out in these parasites by conventional techniques. "Knockout" mutants and reconstitution of the missing genes of the parasite demonstrate the importance of gene products to the varying life-cycle stages of the parasite which are identified using antibodies to proteins and ability to form cysts *in vivo* which define the life cycle stages. The parasites in which a gene is knocked out are a useful basis for an attenuated vaccine. The genes encoding the enzymes or parts of them (e.g., a novel targeting sequence) or the proteins themselves alone or with adjuvants comprise a useful basis for a vaccine. The pathways and enzymes of the invention are useful to design related antimicrobial agents. The sequences and definition of the active sites of these enzymes, and pathways, and organelle (e.g., plastid) targeting sequences provide even more specific novel and unique targets for rational design of antimicrobial agents effective against Apicomplexan parasites. For example, proteins which interact with the enzyme and interfere with the function of the enzyme's active site, or are competitive substrates or products or intracellular antibodies (*i.e.*, with a gene encoding the Fab portion of an antibody that targets the protein the antibody recognizes), or antisense nucleic acid or targeted ribozymes that function as inhibitors are useful, novel antimicrobial agents. Enzymes of the invention are a novel basis for unique diagnostic tests. Because some of these pathways are important in dormant parasites, or in selecting the dormant or active life cycle stages, they are especially important as antimicrobial agent targets for life cycle stages of the parasite for which no effective

antimicrobial agents are known or as diagnostic reagents which ascertain the duration of infection.

Identification of the pathways in Apicomplexan parasites provides additional enzyme targets present in these pathways which are not present in or are differentially expressed in animal cells. Identification of the interrelatedness of these pathways with each other provides the basis for the development and demonstration of combinations of inhibitors which together have an effect which is greater than the expected additive effect (*i.e.*, synergistic). The meaning of synergism is that compound A has effect A', compound B has effect B', compounds A+ B have an effect greater than A'+B'. Synergism is characteristic of inhibitors of these pathways because an initial pathway affected by an inhibitor often provides a product used as a substrate for another pathway so the inhibition of the first enzyme is amplified. These pathways or their products are interrelated. Therefore, the enzymes or DNA which encodes them are targeted by using two or more inhibitors leading to an additive or synergistic effect. Examples include the additive effect of gabaculine and sulfadiazine and the synergistic effects of NPMG and sulfadiazine and NPMG and pyrimethamine. One or more of the inhibitors preferentially affect one of the life cycle stages of Apicomplexan parasites.

Some enzymes are preferentially used by specific stages of the parasites. Detection of an enzyme of this type or a nucleic acid encoding it offers a novel diagnostic test not only for presence of a parasite, but also for identification of the stage of the parasite

Genes encoding enzymes in pathways of the present invention are "knocked out" using techniques known in the art. A parasite with a gene knocked out is said to

be attenuated either because the gene expression of the enzyme is stage specific so the parasite cannot become latent, or because the knocked out enzyme is essential for parasite survival. The importance of an enzyme's functions in various life-cycle stages is determined using a mutant-knockout-complementation system. In the former case, the attenuated parasite is useful as a vaccine because the "knocked out" gene is critical for the parasite to establish latency. Its administration to livestock animals results in immunity without persistence of latent organisms. Mutants with the gene "knocked out" also can be selected because when the parasites are grown *in vitro* they are grown in the presence of product of the enzymatic reaction to allow their survival. However, such attenuated parasites do not persist *in vivo* in the absence of the product and, consequently they are useful as vaccines, for example, in livestock animals. The genes that encode the protein also are used in DNA constructs to produce proteins themselves or the proteins or peptides are used in immunized animals. These constructs are used to elicit an immune response and are used for vaccines alone or with adjuvants. Specific examples are incorporation of the gene for alternative oxidase or chorismate synthase in a construct which has a CMV promoter and expresses the protein following intramuscular injection (i.e., a DNA vaccine). This type of construct. but with genes not identified or described as plant-like, has been used as in a vaccines that protect against bacterial and protozoal infections.

Plant-like pathways in Apicomplexans were inhibited *in vitro*. An Apicomplexan GSAT enzyme that is part of a heme synthesis pathway was targeted with inhibitors A gene with homology to ALA synthase was identified by analysis of the *T. gondii* ESTs (Washington University *T. gondii* gene Sequencing project).

indicating that *T. gondii* has alternative methods for synthesis of ALA. An Apicomplexan glyoxylate cycle was analyzed to determine the sensitivity of tachyzoites and bradyzoites to glyoxylate cycle inhibitors. Specifically, Apicomplexans have isocitrate lyase and malate synthase which present a unique pathway for lipid metabolism that is targeted by inhibitors. Apicomplexan alternative oxidase is targeted, as evidenced by effects of inhibitors of alternative oxidase on this pathway and its expression and immunolocalization in tachyzoites and bradyzoites; Apicomplexan parasites have a metabolically active EPSP synthase enzyme involved in conversion of shikimate to chorismate. These four metabolic pathways, i.e., heme synthesis, shikimate pathway, alternative generation of energy, and the glyoxylate cycle are all exemplified in *T. gondii*. To show that inhibition was specific for key enzymes in these pathways that are generally absent or used only rarely in mammalian cells, product inhibition studies were used *in vitro*. For example, growth of *T. gondii* is sensitive to NPMG that inhibits the synthesis of folic acid via the shikimate pathway. Because mammalian hosts lack the entire shikimate pathway, it is unlikely that the parasites can obtain either PABA or its precursor chorismate from the host cells so provision of PABA circumvents the need for the substrate pathway for folate synthesis and rescues the EPSP synthase inhibition by NPMG.

Further proof of the presence of the plant-like pathways arises from biochemical assays for an enzyme in analogous plant pathways and isolation of encoding genes. Genes are identified by search of available expressed sequence tags (ESTs, i.e., short, single pass cDNA sequences generated from randomly selected library clones), by PCR amplification using primer sequences derived from published conserved sequences of

plant genes with parasite genomic DNA or parasite DNA libraries (Chaudhuri et al., 1996), by the screening of Apicomplexan DNA expression libraries with antibodies to previously isolated homologous proteins or the DNA which encodes them and by complementation of *E. coli* or yeast mutants deficient in an enzyme. Genes isolated by these techniques are sequenced which permits identification of homologies between plant and Apicomplexan genes using sequence databases such as Genbank. These assays confirm that an enzyme and the gene encoding it are present in Apicomplexan parasites. *E. coli* mutants and yeast deficient in the enzyme are complemented with plasmid DNA from *T. gondii* cDNA expression libraries or the isolated gene or a modification (e.g., removing a transit sequence) of the isolated gene which allows the production of a functional protein in the *E. coli* or yeast, demonstrating that the gene encoding the enzyme is functional. Homologous genes in *T. gondii*, *P. malaria*, *Cryptosporidia*, *Neospora*, and *Eimeria* are identified when relevant plant or *T. gondii* genes are used as probes to DNA obtained from these organisms and the genes are identified either by cloning and sequencing the DNA recognized by the probe or by using the probe to screen the relevant parasite libraries. Genomic DNA is sequenced and identifies unique promoters which are targeted. Unique parts of the genes were identified in the sequences and provide additional antimicrobial agent targets, diagnostic reagents and vaccine components or bases for vaccines. Clade and bootstrap analyses (Kohler et al., 1997) establish the phylogenetic origin of novel, sequenced, parasite genes and this indicates other related antimicrobial agent targets based on components, molecules, and pathways of phylogenetically related organisms. Gene products are expressed and utilized for enzyme assays and for screening novel inhibitors, for making

antibodies for isolation of native protein, for x-ray crystallography which resolves enzyme structures and thus establishes structure-function relationships and enzyme active sites which are useful for the design of novel inhibitors.

Immunoelectronmicroscopy using antibodies to enzymes such as chorismate synthase, alternative oxidase, malate synthase or isocitrate lyase immunolocalizes the enzymes within the parasite and determines their location, in particular whether they are in plant-like organelles. Apicomplexan transit peptides are identified by their homology to known transit peptides in other species. Attachment of reporter proteins to the wild type transit peptide, or deletion or mutations of the transit peptide or portion of the peptide or gene encoding it, and then characterization of targeting of these constructs alone or in association with reporter constructs establishes that the amino acid sequences of the transit peptide determine intracellular localization and site of function of proteins with this sequence. Stage specificity of these enzymes is determined *in vitro* by using antibodies to stage-specific antigens in inhibitor-treated cultures, by Western or Northern analyses (detection), by enzyme assays using selected parasite life cycle stages, by using RT PCR (Kirisits, et al. 1996) and a DNA competitor as an internal standard to quantitate the amount of mRNA in parasite samples, by ELISA (quantitation) and by determining whether a parasite with the gene knocked out can develop a bradyzoite phenotype *in vitro* in the appropriate bradyzoite inducing culture conditions. Stage specificity *in vivo* is determined by observing effects of the inhibitors on different life cycle stages in acutely vs chronically infected mice and by determining whether a parasite with the gene knocked out can form cysts *in vivo*. Useful techniques

to develop diagnostic reagents for detection of these proteins or nucleic acids include ELISAs, Western blots, and specific nucleotides used as probes.

EXAMPLES

Example 1: Novel *In Vitro* Assay Systems to Assess Antimicrobial Effects on *T. gondii*

New *in vitro* and *in vivo* assay systems were developed to determine whether plant metabolic pathways are present in Apicomplexans. New elements include use of longer culture times (*e.g.*, extending the duration of the assay to ≥ 6 days is also a unique and useful aspect of this invention, because it allows demonstration of antimicrobial effect for compounds which have to accumulate prior to exerting their effect), use of Me49 PTg and R5 strains *in vitro*, employing synergistic combinations of NPMG and low dosage pyrimethamine *in vivo*, and assays of parasitemia *in vivo* using competitive PCR.

Improvements were developed in the assays reported by Mack *et al.* (1984) and Holfels *et al.* (1994) to measure *T. gondii* replication in tissue culture. The improvements are based on microscopic visual inspection of infected and inhibitor treated cultures, and on quantitation of nucleic acid synthesis of the parasite by measuring uptake of ^{3}H uracil into the parasite's nucleic acid. Uracil is not utilized by mammalian cells. Parasites present as tachyzoites (RH, Ptg, a clone derived from the Me49 strain), bradyzoites (Me49), and R5 mutants (mixed tachyzoite/bradyzoites of the Me49 strain that can be stage switched by culture conditions) (Bohne *et al.*, 1993, Soete *et al.*, 1994; Tomovo and Boothroyd, 1995; Weiss *et al.*, 1992) are suitable for assay systems used to study effects of inhibitors. Only the RH strain tachyzoites, cultured for up to 72 hours, had been used in previously reported assays. The use of

Me49, Ptg, and R5 mutant are unique aspects of the methods used in these assays in this invention.

Results using the assay systems are shown in FIGS. 4, 6-8. In these assays toxicity of a candidate inhibitor was assessed by its ability to prevent growth of human foreskin fibroblasts (HFF) after 4 days and after 8 days as measured by tritiated thymidine uptake and microscopic evaluation. Confluent monolayers of HFF were infected with tachyzoites or bradyzoites. Inhibitor was added one hour later. Non-toxic doses were used in parasite growth inhibition assays. Parasite growth was measured by ability to incorporate tritiated uracil during the last 18 hours of culture.

Example 2: <u>Detection of Plant-like Pathways in Apicomplexans</u>

Using assays disclosed herein, some of which were novel, Apicomplexan parasites were found to contain at least four metabolic pathways previously thought to be unique to plants, algae, bacteria, dinoflagellates, and fungi. Specifically, the presence of a unique heme synthesis pathway, an alternative oxidase pathway, a glyoxylate cycle and a pathway necessary for the biosynthesis of chorismate and its metabolites were explored. Growth of the parasite, *T. gondii*, depends upon these pathways. To examine *T. gondii* for the presence of plant-like and algal metabolic pathways, certain inhibitors of metabolic pathways are suitable to apply because of their ability to prevent growth of the parasite in tissue culture.

Pathways which are present in Apicomplexans were analyzed as follows: First, *T. gondii* tachyzoites were tested to see if they were sensitive *in vitro* to inhibition by specific inhibitors of target pathways Then bradyzoites are tested Positive results for each pathway provided presumptive evidence that the inhibitor targets were present and

that their activities are important for parasite survival and growth. The inhibitors effective *in vitro* were screened for activity *in vivo* in mice. An example of an effective combination *in vivo* is NPMG and low dosage pyrimethamine.

The presence of an enzyme was further confirmed by product rescue *in vitro*, in which the product abrogates the need for its synthesis by the enzyme. An example was rescue by PABA for the reaction catalyzed by EPSP synthase. Other methods to demonstrate the presence of an enzyme and thus the pathway include functional enzyme assays, complementation of mutant *E. coli* strains, PCR, screening of a *T. gondii* expression library with antibodies or DNA probes, and immunostaining of Western blots. For some enzymes, identification of a partial sequence of a gene in an EST library in the gene database led to cloning and sequencing the full length gene. Demonstration of the enzymes also is diagnostic for presence of the parasites. Examples are demonstration of *T. gondii* and *C. parvum* GSAT and *T. gondii* alternative oxidase and *T. gondii* isocitrate lyase and malate synthase by Western analysis and cloning and sequencing of the *T. gondii* and *P. falciparum* chorismate synthase gene. Reagents (gene probes and antibodies) obtained during characterization of genes from *T. gondii* are used to detect homologous enzymes and pathways in other Apicomplexan parasites. Examples were using the *T. gondii* chorismate synthase gene to probe *P. falciparum*, *Eimeria bovis* and *Cryptosporidium parvum* genomic DNA. Other examples are using heterologous plant DNA to detect Apicomplexan GSAT, isocitrate lyase, malate synthase, and alternative oxidase genes. Such genes are used as DNA probes to screen libraries to clone and sequence the genes to identify PCR products

Example 3: Effects of Inhibitors *In Vitro* on *T. gondii*

Using the assays described in Example 1, five compounds that restrict the growth of *T. gondii in vitro* were identified:

(i) Gabaculine

(ii) NPA

(iii) SHAM (Salicylhydroxamic Acid);

(iv) 8-hydroxyquinoline

(v) NPMG

Specifically these inhibitors act as follows:

i. **The Effect of Gabaculine, An Inhibitor Of The 5-Carbon Heme Synthesis Pathway, On the Growth of *T. gondii***

FIG. 1A compares heme biosynthesis in plants, algae and bacteria with heme biosynthesis in mammals. In higher plants and algae, ALA is produced in the plastid by the action of GSA aminotransferase on glutamate 1-semialdehyde. In mammals, ALA is formed through the condensation of glycine and succinyl CoA. ALA is subsequently converted to heme. In one dinoflagellate and *T. gondii* both pathways are present.

Inhibitors of plant heme synthesis pathway restrict the growth of *Toxoplasma gondii in vitro*. As shown in FIG. 1A, the synthesis of δ-aminolevulinic acid (ALA), the common precursor for heme biosynthesis, occurs in the plastid of plants, algae and Apicomplexan parasites by the 5-carbon pathway and ALA synthesis occurs by a different pathway in animals. The pathway in animals involves the condensation of glycine and succinyl CoA. The data in FIG. 1B-C and a Western blot utilizing an antibody to the homologous soybean, and barley, and synechococcus GSATs,

demonstrate that *Toxoplasma gondii* utilizes the 5-carbon pathway for ALA synthesis and therefore heme biosynthesis. 3-amino 2,3-dihydroxybenzoic acid (gabaculine) inhibits GSA in the heme synthesis pathway.

First the toxicity of gabaculine was assessed by its ability to prevent growth of human foreskin fibroblasts (HFF) as measured by ^{3}H-thymidine uptake and microscopic evaluation. Non-toxic doses were used in parasite growth inhibition assays. *In vitro* parasite growth inhibition assays included confluent monolayers of HFF infected with tachyzoites (RH) or mutant Me49 (R5). Gabaculine was added 1 hour later. Parasite growth was measured by the ability to incorporate ^{3}H-uracil during the last 18 hours of culture. In addition, parasite growth was evaluated microscopically in Giemsa stained slides.

Toxoplasma organisms were grown in human foreskin fibroblasts alone and in the presence of different concentrations of gabaculine (3-amino-2,3-dihydrobenzoic acid). Growth was measured by the ability of *T. gondii* to incorporate tritiated uracil. This compound was effective at inhibiting the growth of *T. gondii* at the 20mM concentration. FIG. 1B demonstrates the ability of gabaculine (a specific inhibitor of GSA aminotransferase) to restrict the growth of *T. gondii* in an *in vitro* assay over a 4 day period. *T. gondii* growth is measured by ability of the parasites to incorporate tritiated uracil and is expressed as counts/minute (CPM) on the Y-axis. The X-axis describes how the *T. gondii* cultures were treated. Cultures that were grown in medium (medium) produced a CPM of around 45,000. If no *T. gondii* were added to the cultures (no RH), a CPM of around 2,000 was observed. Pyrimethamine (0.1 μm/ml) and sulphadiazine (12.5 μg/ml) added to cultures resulted in a marked

reduction in CPM compared with untreated cultures. At a dose of 5 mM gabaculine restricted around 50% of CPM and at a dose of 20 mM it almost completely inhibited parasite growth, with counts of about 5,000 CPM.

FIG. 1C demonstrates the ability of gabaculine to inhibit the growth of *T. gondii* over 8 days in culture. *T. gondii* growth is measured by ability of the parasites to incorporate tritiated uracil and is expressed as counts/minute (CPM) on the Y-axis. The X-axis represents days post infection.. Parasite growth was evident in the cultures where no drug was added (medium) over the entire time course. Parasite growth was restricted in cultures with 20 mM gabaculine (gabaculine) over the 8 day time course. Similarly, parasite growth was restricted in cultures with pyrimethamine and sulphadiazine (P/S) over the 8 day time course. Similar concentrations showed no toxicity to the foreskin fibroblasts indicating the specificity of this compound for *T. gondii*. Parallel cultures, fixed and stained with Giemsa and examined by microscopy, clearly demonstrated that *T. gondii* growth was substantially inhibited in the presence of 3-amino-2,3-dihydrobenzoic acid. The results in FIGS. 1B and 1C indicate that *T. gondii* utilizes the 5-carbon ALA synthesis pathway.

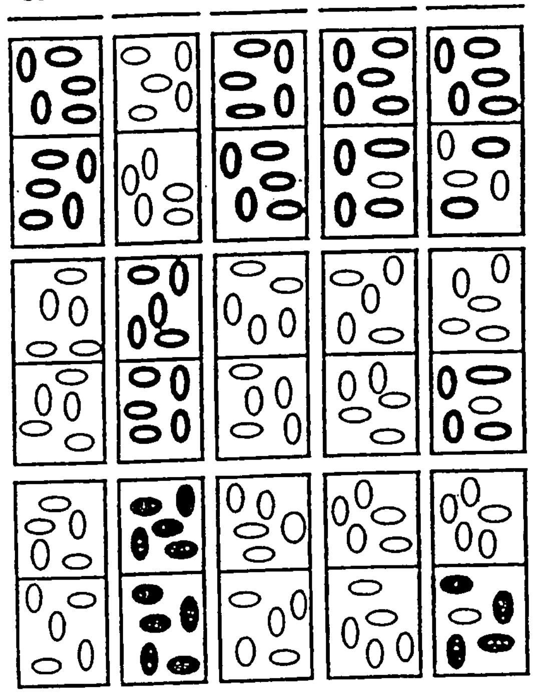
FIG. 7 demonstrates the ability of gabaculine to inhibit the growth of the mutant R5 strain of *T. gondii* over 8 days in culture. This mutant strain is atovaquone resistant and possesses certain characteristics of the tachyzoite stage and certain characteristics of the bradyzoite stage. *T. gondii* growth is measured by their ability to incorporate tritiated uracil and is expressed as counts/minute (CPM) on the Y-axis The X-axis represents days post infection Parasite growth was evident in the cultures where no drug was added (medium) over the entire time course. Parasite growth was

restricted in cultures with 20mM gabaculine (gabaculine) over the first 6 days of culture, after which a marked increase in parasite growth was detected. Furthermore groups of proliferating organisms which resembled tissue cysts were observed in similarly treated cultures. Parasite growth was restricted in cultures with pyrimethamine and sulphadiazine (P/S) over the entire 8 day time course. Residual R5 organisms in treated cultures at 8 days begin to incorporate uracil again and some of them appeared cyst-like. Therefore, *T. gondii* cyst-like structures are selected by gabaculine treatment of cultures. Specific immunostaining of such cultures treated with gabaculine for tachyzoite and bradyzoite specific antigens demonstrates that gabaculine selects bradyzoites. Table 2 is a schematic representation of experiments designed to test the hypothesis that tachyzoites utilize both conventional oxidase and alternative oxidases, but bradyzoites only use alternative oxidases, therefore interfering with generation of iron sulfated proteins by gabaculine treatment will select bradyzoites. The design and predicted results of stage specific immunostaining (Kasper *et al.*, 1983) if the hypothesis were correct are shown in Table 2 and confirm the hypothesis. These results suggest that *T. gondii* has stage-specific utilization of alternative oxidases which are utilized when cell cultures are treated with gabaculine because it depletes heme and thus depletes iron sulfated proteins used in conventional respiration.

In summary, 3-amino-2,3-dihydrobenzoic acid (gabaculine) is an inhibitor of the 5 carbon heme synthesis pathway present in Apicomplexan parasites. Heme synthesis occurs by a different pathway in mammalian cells and is therefore unaffected by 3-amino-2,3-dihydrobenzoic acid

Table 2. Gabaculine treatment of cultures selects bradyzoites.

Antibody used for IFA	Treatment of culture	Tachyzoite Control	Bradyzoite Control	IFA result on culture day 0	2	6
α SAG1 (expressed on tachyzoites only)	Media					
	Gabaculine					
α BSAG (expressed on bradyzoites one day after stage switch)	Media					
	Gabaculine					
α BAG5 (expressed on bradyzoites by five day after stage switch in culture)	Media					
	Gabaculine					



IFA is immunofluorescent assay. SAG1 is surface antigen 1. BSAG is bradyzoite surface antigen 1. BAG5 is bradyzoite antigen 5. A. Hypothesis. B. Design and predicted results of stage specific immunostaining if hypothesis were to be correct. Indicates no specific fluorescence of the organism; indicates specific surface fluorescence of the organism due to presence of the antigen recognized by the antibody (e.g., αSAG1 or αBSAG); Indicates specific internal fluorescence in the organism due to presence of the antigen within the parasite recognized by the antibody (e.g., αBAG5).

ii. An inhibitor of the glyoxylate cycle restricts the growth of *T. gondii in vitro.*

3-Nitropropionic acid is an inhibitor of isocitrate lyase in the degradation of lipid to C4 and inhibits replication of *T. gondii in vitro*. FIG. 2A illustrates how the glyoxylate cycle manufactures C4 acids. Acetyl CoA, a byproduct of lipid breakdown combines with oxaloacetate to form citrate. By the sequential action of a series of enzymes including isocitrate lyase, succinate is formed. Glyoxalate, the byproduct of this reaction is combined with a further molecule of acetyl CoA by the action of malate synthase. Malate is then converted to oxaloacetate, thus completing the cycle. 3-NPA and itaconic acid are inhibitors of this pathway. FIG. 2B demonstrates the ability of 3-NPA (an inhibitor of isocitrate lyase) to restrict the growth of *T. gondii* in an *in vitro* assay over a 4 day period. This result indicates it is likely that *T. gondii* degrades lipids using isocitrate lyase. *T. gondii* growth is measured by their ability to incorporate tritiated uracil and is expressed as counts/minute (CPM) on the Y-axis. The X-axis described how the *T. gondii* cultures were treated. Cultures that were grown in medium (medium) produced a CPM of about 30,000. If no *T. gondii* were added to the cultures (no RH), a CPM of about 2,000 was observed. Pyrimethamine (0.1 μg/ml) and sulphadiazine (12.5 μg/ml) added to cultures resulted in a marked reduction in CPM compared with untreated cultures. A dose of 0.006 mg ml 3-NPA (3-NPA) restricted around 60% of CPM 3-NPA inhibits the glyoxylate cycle (isocitrate lyase) and/or succinate dehydrogenase in Apicomplexan parasites.

iii. and iv. Effect of SHAM and 8-hydroxyquinoline on alternative oxidase in *T. gondii*

There is a metabolic pathway found in most plants and algae and in Apicomplexans, but absent in most multicellular animals. FIG. 3A describes the electron transport respiratory chain that normally occurs on the inner membrane of mitochondria. In animals, NADH and succinate produced by the action of the citric acid cycle diffuse to the electron transport chain. By a series of oxidation reactions mediated in part through the cytochromes, free energy is released. This free energy yields the potential for the phosphorylation of ADP to ATP. In plants, in addition to the conventional electron transport chain complexes, there is an alternative pathway of respiration. Alternative pathway respiration branches from the conventional pathway at ubiquinone and donates released electrons directly to water in a single four electron step. An important feature of this pathway is that it does not contribute to transmembrane potential and thus free energy available for the phosphorylation of ADP to ATP. The pathway provides a source of energy and is preferred for conditions with relatively low ATP demands. A key enzyme in this pathway is an alternative oxidase that is cyanide insensitive and does not require heme. *Toxoplasma gondii* utilizes the alternative oxidase for respiration.

FIG. 3B demonstrates the ability of SHAM (a specific inhibitor of alternative oxidase) to restrict the growth of *T. gondii* in an *in vitro* assay over a 4 day period. The ability of these compounds to inhibit the growth of *T. gondii* was examined by the assay described in Example 1. *T. gondii* growth is measured by their ability to incorporate tritiated uracil and is expressed as counts/minute (CPM) on the Y-axis.

The X-axis describes how the *T. gondii* cultures were treated. Cultures that were grown in medium (medium) produced a CPM of around 54,000. If no *T. gondii* were added to the cultures (no RH), a CPM of around 1,000 was observed. Pyrimethamine (0.1 µg/ml) and sulphadiazine (12.5 µg/ml) added to cultures resulted in a marked reduction in CPM compared with untreated cultures. A dose of 0.19 µg/ml SHAM (0.19) restricted around 50% of CPM and at a dose of 0.78 µg/ml it essentially inhibited parasite growth, with counts of about 8,000 CPM.

Salicylhydroxamic acid (SHAM) and 8-hydroxyquinoline are inhibitors of the alternative oxidase and are also effective against *T. gondii*, presumably by inhibiting the alternative pathway of respiration. Salicylhydroxamic acid and 8-hydroxyquinoline inhibit the alternative oxidase of *T. gondii* tachyzoites. Since alternative oxidative respiration does not occur in mammals, this makes antimicrobial compounds targeting this pathway therapeutic candidates.

v. Effect of NPMG

The shikimate pathway is common to plants, fungi and certain other microorganisms and Apicomplexan parasites, but it is not present in mammalian cells. FIG. 4A details the events that result in the production of tetrahydrofolate, aromatic amino acids and ubiquinone in plants, algae, bacteria and fungi. In this pathway, chorismate is formed through the sequential action of a number of enzymes including EPSP-synthase and chorismate synthase. EPSP-synthase is inhibited by NPMG. Chorismate is further processed to yield tetrahydrofolate or ubiquinone by a further series of enzymatic reactions. This pathway has not been described in mammals which are dependent on diet for folate and therefore for tetrahydrofolate production. This

pathway is required for the synthesis of certain aromatic amino acids and aromatic precursors of folic acid and ubiquinone. It is likely that *Toxoplasma gondii* utilizes the shikimate pathway for synthesis of folic acid, ubiquinone and aromatic amino acids.

N-(phosphonomethyl) glycine, an inhibitor of 3-phospho-5-enolpyruvylshikimate (EPSP) synthase and thus an inhibitor of shikimate to chorismate conversion, affects the pathway (Table 1). the ability of this compound to inhibit the growth of *T. gondii* was examined by the assay described in Example 1.

FIG. 4B demonstrates the ability of NPMG (a specific inhibitor of EPSP-synthase) to restrict the growth of *T. gondii* in an *in vitro* assay over a 4 day period. *T. gondii* growth is measured by their ability to incorporate tritiated uracil and is expressed as counts/minute (CPM) on the Y-axis. The X-axis describes how the *T. gondii* cultures were treated. Cultures that were grown in medium (medium) produced a CPM of around 72,000. If no *T. gondii* were added to the cultures (no RH), a CPM of around 2,000 was observed. Pyrimethamine (0.1 μg/ml) and sulphadiazine (12.5 μg/ml) added to cultures resulted in a marked reduction in CPM compared with untreated cultures. At a dose of 3.12 mM NPMG (3.12) restricted around 60% of CPM and at a dose of 4.5 mM it inhibited parasite growth by around 80%, with counts of about 12,000 CPM.

In FIG. 4C the ordinate shows uptake of tritiated uracil into *T. gondii* nucleic acids, inhibitory effects of NPMG on nucleic acid synthesis is shown; where PABA at increasing concentrations is added to such cultures, PABA abrogates the inhibitory effects of NPMG on EPSPS synthase restoring nucleic acid synthesis.

vi. Branched Chain Amino Acid Synthesis

Imidazolinones and sulfonylureas inhibit acetohydroxy acid synthase in Apicomplexan parasites.

vii. Starch (amylopectin) Synthesis and Degradation

UDP glucose starch glycosyl transferase is inhibited by substrate competition in Apicomplexan parasites.

viii. Transit Sequences

Antisense, ribozymes, catalytic antibodies, (Pace et.al., 1992; Cate et al., 1996; Charbonnier 1997; Askari et.al., 1996) conjugation with toxic compounds allow targeting of parasite molecules using transit sequences.

Identification of transit sequences in Apicomplexans provides many means for disruption of metabolic pathways. Antisense or ribozymes prevent the production of the transit peptide and associated protein. Alternatively production of transit peptide sequences, and the conjugation to toxic molecules, allow disruption of organellar function. Catalytic antibodies also are designed to destroy the transit sequence. These antisense compounds or ribozymes or toxic molecules targeted to transit sequences with intracellular antibodies are used as medicines to inhibit the parasite.

Example 4: Plant-like Pathways and Enzymes in Apicomplexan Parasites

Plasmodium falciparum and *Cryptosporidia parvum*

Based on the effects of inhibitors of plant-like pathways, abrogation of inhibitor effects, and detection of specific enzymes and/or genes, Apicomplexans, in general, have plant-like pathways. Results shown in this example broaden the observations of

the presence of plant-like pathways in Apicomplexans beyond the representative parasite *T. gondii*.

i. **Heme Synthesis**

Gabaculine inhibited the heme synthesis pathway (GSAT) in Apicomplexan parasites (FIGS. 1B and 1C, *T. gondii*; FIG. 6, *Cryptosporidia)* but with modest or no affect on *P. falciparum* (Table 3, *Malaria*).

FIG. 6 demonstrates the effect of NPMG, gabaculine, SHAM and 8-hydroxyquinoline and 3-NPA on *Cryptosporidia in vitro*. *C. parvum* oocysts at 50,000/well were incubated at 37° C (8% CO_2) on confluent MDBKF5D2 cell monolayers in 96 well microtiter plates with the following concentrations of each drug. The concentrations used were: SHAM (0.2% ETOH was added) 100, 10, 1, 0.1 µg/ml; 8-hydroxyquinoline 100, 10, 1, 0.1 µg/ml; NPMG 4.5, 0.45, 0.045 µg/ml; gabaculine 20, 2, 0.2 µg/ml. The level of infection of each well was determined and analyzed by an immunofluorescence assay at 48 hours using an antibody to *C. parvum* sporozoites made in rabbits at a concentration of 0.1%. Fluorescein-conjugated goat anti-rabbit antibody was used at a concentration of 1%. 95% CI count was the mean parasite count per field when 16 fields counted at 10x magnification ± s.d. of the mean. The approximate 95% CI counts were as follows: media and ethanol ~ 1200; paromomycin (PRM) and ethanol ~ 100; SHAM 100 µg/ml ~ 400; SHAM 10 µg/ml ~ 1100; SHAM 1 µg/ml ~ 1100, SHAM 0.1 µg/ml ~ 1200; media alone ~ 1800 µg/ml; PRM ~200; 8-OH-quinoline 100 µg/ml; ~300; 8-OH-quinoline 10 µg/ml; ~ 900, 8-OH-quinoline 1 µg/ml ~1100, 8-OH-quinoline 0.1 µg/ml ~ 1300; NPMG

4.5 µg/ml ~ 900; NPMG 0.45 µg/ml ~ 1200; NPMG 0.045 ~ 1200; gabaculine 20 µg/ml ~ 200; gabaculine 2 µg/ml ~600; and gabaculine 0.2 µg/ml ~ 1300. Thus each of these compounds are promising lead compounds as antimicrobial agents effective against *Cryptosporidia*.

ii. **Glyoxylate cycle**

3-NPA inhibited the glyoxylate cycle (isocitrate lyase) and/or succinate dehydrogenase in Apicomplexan parasites (**FIG. 2B**, *T. gondii)* and also inhibited *P. falciparum* and *C. parvum*.

To determine whether there is an Apicomplexan glyoxylate cycle, to analyze the sensitivity of *T. gondii* tachyzoites and bradyzoites to glyoxylate cycle inhibitors and to determine whether Apicomplexan parasites have isocitrate lyase which presents a unique pathway for lipid metabolism that can be targeted with inhibitors, the following methods are suitable.

The inhibitor of isocitrate lyase is 3-nitropropionic acid (concentration ranging from 0.005 to 5mg/ml *in vitro*, and 5 to 50 mg/kg/day *in vivo*). Mutants [Yale Stock Center] used for complementation are as follows: *E. coli* strains; DV 21A01 (aceA which lacks isocitrate lyase) and DV21 A05 (aceB which lacks malate synthase). Plant gene sequences suitable for comparison are those described by Kahn *et al.* (1977), Maloy *et al.* (1980); and Maloy *et al.* (1982). A biochemical assay for isocitrate lyase activity is the method of Kahn *et al.* (1977). The polyclonal antibodies to cotton malate synthase and cotton isocitrate lyase which hybridize to *T. gondii* proteins of

approximately 60 kd are used to identify these enzymes in other Apicomplexan parasites.

iii. Alternative Oxidase

SHAM and 8-hydroxyquinoline inhibited the alternative pathway of respiration, *i.e.*, the alternative oxidase in Apicomplexan parasites [FIG. 3, *T. gondii;* FIG. 6, *Cryptosporidia parvum;* Table 3, *Plasmodium falciparum* (clones W2, D6), pyrimethamine resistant or sensitive clones. Because *Cryptosporidia* appear to lack mitochondria, the plastid is a likely site for the alternative pathway of respiration.

Table 3. Effect of NPMG, SHAM, 8-OH quinoline, 3NPA and gabaculine on the D6 and W2 clones of *Plasmodium falciparum**

Inhibitor	Parasite Clone	Conc (ng/ml)	
		IC 50	IC 90
NPMG	D6 W2	823 1716	2510 3396
SHAM	D6 W2	6210 5705	25066 42758
8-OH-quinoline	D6 W2	1204 1631	1883 4521

*Assays were performed in accordance with Milhous *et al.*, 1985; Odula *et al.*, 1988. Concentrations (ng/ml) of other compounds that inhibited these clones in this assay were as follows for the W2 and D6 clones: Pyrimethamine (82.10, 0.05), Chloroquin (40.86, 2.88), Quinine (38.65, 4.41), HAL (0.33, 0.51), Atovaquovone (0.13, 0.12). 3NPA also inhibited *P. falciparum* with IC 50=3304, 2817; IC 90=4606, 2817 but with a very small or no significant effect of gabaculine (IC 50 ≥ 45,000).

Effect of SHAM on wild type malaria *in vitro* had been described earlier (Fry and Beesley, 1991). However, this observation was presented without knowledge that SHAM affected alternative oxidase function.

iv. Shikimate/Chorismate

NPMG inhibited the shikimate pathway in Apicomplexan parasites (FIG. 4B, *T. gondii*, Table 4, *Malaria;* FIG. 6, *Cryptosporidia*)

Presence of a product of the enzymatic reaction in the pathways of the present invention abrogates the effect of the inhibitor on a specific enzyme because the product no longer has to be made by enzyme catalysis of a substrate. Thus, addition of the product proves the specificity of the effect of the inhibitor on the enzyme. The addition of PABA abrogates the exogenous effect of NPMG which is an inhibitor of EPSP synthase (FIG. 4B, *T. gondii*). Because PABA ablates the effect of the inhibitor NPMG on EPSP synthase, the presence of the shikimate pathway in Apicomplexan parasites is demonstrated.

Other specific methods to determine whether Apicomplexan parasites have a metabolically active EPSP synthase enzyme involved in conversion of shikimate to chorismate and further characterize this metabolic pathway in *T. gondii* are as follows:

Use of the inhibitor N-(phosphonomethyl) glycine (concentrations of 3.125mM *in vitro* and 100 mg/kg/day *in vivo*). The product rescue assays are performed with PABA. The mutants for complementation are as follows: *E. coli, AroA*; *E. coli, AroC*; and yeast, *AR*. [Yale Stock Center] Plant gene sequences for comparison are outlined by Klee *et al.* (1987). A biochemical assay for EPSP synthase activity in cellular lysates is as described by Mousdale and Coggins (1985). Other enzymes in this pathway also are characterized (Nichols and Green, 1992). The full length nucleotide sequence of chorismate synthase was obtained following restriction digestion and primer-based sequencing of the Tg EST zyllc05.r1 clone obtained from the "Toxoplasma EST Project at Washington University" and of *P. falciparum* EST czap PFD d2.1 clone obtained from the "malaria EST project." D Chakrabarti, Florida. The *Toxoplasma gondii* sequence has substantial homology with tomato and several other chorismate

synthases and a region of the *T. gondii* protein has 30% identity and 45% homology with the transit sequence of *Zea mays* (sweet corn). Other inhibitors of EPSP synthase are Inhibitors 4 and 5, sulfosate (Marzabadi et al., 1996). Other inhibitors of enzymes in this pathway also have been developed by others and provide a paradigm for the rational synthesis of competitive substrate inhibitors of Apicomplexan parasites.

v. Branched Chain Amino Acid and Other Essential Amino Acid Synthesis

Acetohydroxy acid synthase is an enzyme present in plants but not animals and is inhibited by imindazolinones and sulfonylureas in Apicomplexan parasites. Inhibitors of histidine synthesis restrict growth of Apicomplexan parasites.

vi. Starch (Amylose/Amylopectin) Synthesis and Degradation

UDP glucose starch glycosyl transferase, starch synthetase and Q (branching) enzymes are inhibited by substrate competitors in Apicomplexan parasites.

vii. Lipid Synthesis

The plant-like acetyl coA decarboxylase is inhibited by a number of inhibitors shown in Table 1B. Linoleic acid and linolenic acid synthases are inhibited by newly designed competitive substrates.

viii. Auxins and Gibercellins

The known auxin mimics and Gibercellin synthesis and Gibercellin inhibitors inhibit Apicomplexan parasites' growth.

ix. Glutamine/Glutamate Synthesis

Glufosinate inhibits Apicomplexan glutamine/glutamate synthesis because the critical enzyme is plant-like.

x. Transit Sequence

The transit sequence is conjugated with toxic molecules such as ricins and used to disrupt plastid function in Apicomplexans. Other strategies, such as antisense, ribozymes or the use of catalytic antibodies prevent translation of DNA to protein or catalyze the destruction of mature protein. This interferes with functioning of the molecule and thus the parasite's growth and survival.

Example 5: <u>The Combined Effects of Inhibitors of Apicomplexan Parasites</u>

The effect of enzymes in pathways "in parallel" are additive and in "series" are more than the additive effect of either inhibitor used alone (*i.e.*, synergistic). FIG. 5 demonstrates the inter-relationship of the shikimate pathway and heme synthesis with the electron transport chain. The shikimate pathway produces 3,4-dihydroxybenzoate which is converted to ubiquinone, an essential component of the electron transport chain. Thus, NPMG, an inhibitor of EPSP-synthase, indirectly affects ubiquinone production and, thus, the electron transport chain. Similarly, heme is required for the production of cytochromes in the electron transport chain. Thus, inhibition of heme production by gabaculine also indirectly affects the conventional electron transport chain. This scheme allows synergistic combinations of drugs. Thus, NPMG and sulphadiazine (a competitive PABA analogue) which act at different points of the folate synthesis pathway are predicted to be synergistic, whereas the effects of gabaculine and sulphadizine (a competitive PABA analogue) which act on different pathways, are

predicted to be additive. Similarly, gabaculine and SHAM are a predicted synergistic combination of inhibitors. Table 4 demonstrates the additive inhibitory effect of sulphadiazine and gabaculine on the growth of *T. gondii* over 4 days in culture. *T. gondii* growth is measured by their ability to incorporate tritiated uracil and is expressed as counts/minute (CPM). Cultures that were grown in medium (medium) produced a CPM of about 36,000. If no *T. gondii* were added to the cultures (no RH). a CPM of about 2,000 was observed. Pyrimethamine (0.1 μg/ml) and sulphadiazine (12.5 μg/ml) added to cultures resulted in a marked reduction in CPM compared with untreated cultures. The growth of *T. gondii* was inhibited by about 60% in cultures treated with 5 mM gabaculine (gabaculine). The growth of *T. gondii* in cultures treated with sulphadiazine (1.56 μg/ml) was reduced by approximately 60%. When this dose of sulphadiazine was used in combination with 5 mM gabaculine, as expected, the combined effect of gabaculine plus sulfadiazine is additive because the pathways are in parallel. In contrast, NPMG and sulfadiazine combine in a synergistic manner. Because heme is needed for conventional mitochondrial respiration, it is expected that if both the heme synthesis and alternative oxidase pathways are present, then 3-amino-2,3-dihydrobenzoic acid and SHAM will demonstrate synergy. Similarly, ubiquinone or end products of the shikimate pathway are needed for mitochondrial respiration and NPMG plus SHAM therefore demonstrate synergy. Table 4 also shows that; the effects of gabaculine and SHAM are not synergistic as would be predicted by this simple model The likely reason for this is that ALA synthase is present in *T. gondii* and provides a default pathway for the synthesis of δ-aminolevulinic acid. Thus, the effects of gabaculine plus SHAM are not synergistic Cycloguanil which affects the

plant-like DHFR-TS of *T. gondii* (McAuley et al. 1994) also is synergistic with NPMG and other inhibitors of enzymes in the shikimate pathway which provides an improved, novel method to treat this infection. Use of synergistic combinations provide an improved strategy for the development of new medicines for the treatment of disease and eradication of the parasite.

Table 4. Representative Effects on Inhibitors Alone and Together on Replication of *T. gondii* which demonstrate synergy

Drug A	Drug B	CPM untreated	CPM for A	CPM for B	CPM for A + B		Ratio
					Actual	Predicted	Actual: Predicted*
NPMG	Sulfadiazine	71449±3763	28138±2216	25026±4365	2368±418	9856	0.24
NPMG	Pyrimethamine	64343±1222	25097±1398	69217±3253	9354±2126	25097	0.37
NPMG	SHAM	64343±1222	25097±1398	42993±1098	7554±970	16769	0.45

Predicted CPM for Drug A + Drug B (if effect is only additive, not synergistic) is calculated as (CPM Drug A x CPM Drug B)/CPM of untreated culture. Concentrations were: NPMG (3.25mM); Sulfadiazine (6.25μg/ml); Pyrimethamine (0.025μg/ml); SHAM (0.78μg/ml).

* A ratio of Actual:Predicted of <1 is considered synergistic. A ratio of Actual:Predicted ≥ to 1 is considered . additive.

Example 6: Effects of Inhibitors *In Vivo*

Candidate inhibitors are administered to animals by daily intraperitoneal injection or by addition to the drinking water. To inhibit EPSP synthase, *in vivo*, NPMG is administered at a dose of 100mg/kg/day.

a) Survival: Five hundred tachyzoites of the RH strain are administered intraperitoneally to BALB/c mice. Cumulative mortality is followed in groups of mice given inhibitor compared to untreated controls.

b) Formation of Cysts: C3H/HeJ mice that have been infected perorally with the Me49 strain of *T. gondii* for 30 days are treated with the inhibitor for 30 days. Cyst burden and pathology in the brains of inhibitor-treated and control mice are compared using methods described previously (Roberts, Cruickshank and Alexander, 1995; Brown *et al.*, 1995; McLeod, Cohen, Estes, 1984; McLeod *et al.*, 1988). Cyst numbers present in a suspension of brain are enumerated, or cyst numbers in formalin fixed paraffin embedded sections are quantitated.

c) Persistence of Cysts: C3H/HeJ mice are infected orally with 100 cysts of *T. gondii* (Me49 strain). Inhibitors are administered to groups of mice from day 30 post infection to day 50 post infection. Cyst burden, mortality and pathology are compared in treated and control mice on days 30 and 50 post infection and in mice that receive antibody to gamma interferon which leads to recrudescence of disease.

d) Synergy: If marked synergistic effect is demonstrated *in vitro* by showing that the subinhibitory concentrations used together exert an effect greater than

the additive effects of each used separately, for any combinations, their effect alone and together *in vivo* is compared.

e). New Assays Which Determine the Effects of Antimicrobial Agents on *T. gondii In Vivo*

Previously reported assay systems measure protection against death following intraperitoneal infection if an animal is infected with the virulent RH strain of *T. gondii*. Novel aspects of the assay systems in the present invention are using the Me49 (AIDS repository) strain of *T. gondii* to determine the effect on brain cyst number following acute peroral infection by an Apicomplexan parasite, the effect on the established number of brain cysts during subacute/chronic infection, and use of the Me49 and RH strains to demonstrate synergy of inhibitors of plant-like pathways of the present invention which are "in series," and a novel system to demonstrate reduction of parasitemia which is quantitated using a competitive PCR technique. In this competitive PCR method the *T. gondii* B1 gene is amplified by PCR in the presence of a construct which produces a product slightly smaller than the wild type B1 gene. The amount of construct can be quantitated to semiquantitate the amount of the competing wild type gene. For example, presence of a greater amount of the wild type gene will result in lesser use of the competitor.

f). Effect of Antimicrobial Agents on Apicomplexan Parasites *In Vivo*

A demonstration of the effect of inhibitors of plant-like metabolic pathways *in vivo* is the synergistic effect of NPMG and low dosage pyrimethamine. NPMG is an inhibitor of infection and promotes survival of mice infected with the virulent RH strain

of *T. gondii* when utilized in conjunction with a low dose of pyrimethamine, whereas neither low dosage pyrimethamine nor NPMG alone are protective. Sulfadiazine reduced manifestations of infection *in vivo*. SHAM affects parasitemia and number of brain cysts.

FIG. 8 demonstrates the ability of NPMG and pyrimethamine administered in combination to protect mice from an otherwise lethal challenge with the virulent RH strain of *T. gondii*. Mice were infected intraperitoneally with 500 tachyzoites and left untreated (control) or treated by the addition of pyrimethamine (PYR), NPMG (NPMG) or both pyrimethamine and NPMG (PYR/NPMG) to their drinking water. Percent survival is marked on the Y-axis and days post infection on the X-axis. Untreated mice and those treated with either pyrimethamine or NPMG died between day 7 and 9 post infection. In contrast 66 percent of mice treated with pyrimethamine and NPMG survived until day 9 post infection and 33 percent survived until the conclusion of the treatment (day 30 post infection). After the withdrawal of treatment, all of these mice survived until the conclusion of the experiment (day 60 post infection).

Example 7: <u>Presence of an Enzyme in a Specific Life Cycle Stage Predicts Efficacy of Inhibitors of the Enzyme on this Stage of the Parasite</u>

The effect of candidate inhibitors on different life cycle stages and their effect on stage conversion is of considerable interest and clinical importance. The bradyzoite form of *T. gondii* was studied by electron microscopy and was found to have a plastid Intraparasite immunolocalization of the enzymes is also performed. Gabaculine treated cultures are stained with antibodies to tachyzoites and bradyzoites. Tachyzoites of the

RH strain are grown in the peritoneum of ND4 mice for 3 days. Tachyzoites are harvested in saline (0.9%) from the peritoneal cavity of euthanized mice and purified by filtration through a 3μm filter. Bradyzoites are isolated as described herein in the Material and Methods. The tachyzoites are pelleted by centrifugation and the pellet is fixed in 2.5% glutaraldehyde. Cysts and bradyzoites are purified from the brains of C57BL10/ScSn mice as described herein in the Materials and Methods and then fixed in 2.5% glutaraldehyde.

Immunoelectronmicroscopy is as described by Sibley and Krahenbuhl (1988) using gold particles of different sizes with antibodies to the enzymes to identify the enzyme localization in different organelles which are identified morphologically. Immunoelectronmicroscopy localization is accomplished with Amersham Immunogold kit and cryosectioning using standard techniques in the electronmicroscopy facility at the University of Chicago or at Oxford University, Oxford, England. Extracellular organisms are studied as well as tachyzoites and bradyzoites at intervals after invasion. Morphology of the parasites, their ultrastructure and the localization of the intracellular gold particles conjugated to the antibodies is characterized. Invasion is synchronized by placing tachyzoites and bradyzoites with PS15 cells at 4°C, then placing cultures at 37°C. Intervals to be studied are before 1, 5, and 10 minutes and 4 hours after invasion.

Immunostaining and immunoelectronmicroscopy using an antibody to soybean, or synechococcus, or barley GSAT indicate whether the enzyme is present or absent in

both the tachyzoite and bradyzoite life cycle stages and localizes the enzyme in the parasite.

a) Immunostaining for tachyzoites and bradyzoites

Immunostaining of tachyzoites and bradyzoites is evaluated with fluorescent microscopy. This is performed on cultures of fibroblasts in Labtech slides infected with tachyzoites (RH strain) or bradyzoites and permeabilized using triton, or saponin or methanol, as described by Weiss *et al.*, 1992; Dubremete and Soete, 1996; Bohne *et al.* (1996). Slides are stained 1, 2, 4, 6, and 8 days post infection with anti-BAG (Weiss *et al.*, 1992) and anti-SAG1 (Mineo *et al.*, 1993; McLeod *et al.*, 1991; Roberts and McLeod, 1996).

b) Antibodies

Antibodies to the bradyzoite antigens (Weiss *et al.*, 1992; and Bohne *et al.*, 1993) and monoclonal and polyclonal antibodies to SAG1 (Kasper *et al.* 1983) as a marker for tachyzoite stage specific antigens are used for immunostaining of parasites to establish stage of the parasite. Transgenic parasites with bradyzoite genes with reporter genes are also useful for such studies.

c) Inhibitors and Stage Switching

The effect of inhibitors of conventional (KCN, Rotenone, Antimycin A or Myxothiazol) respiration and alternative respiration on inhibition of growth of tachyzoites and bradyzoites are compared using standard inhibition experiments in conjunction with immunostaining techniques. Tachyzoites use conventional and alternative pathways of respiration whereas the bradyzoite stage relies on alternative

respiration. Inhibitors of conventional respiration favor tachyzoite to bradyzoite switching whereas inhibitors of alternative respiration inhibit tachyzoite and bradyzoite stages.

d) Synergy studies, gabaculine treatment

Synergy studies with gabaculine are of particular interest because heme is used in the conventional oxidase pathway. If there is synergy, iron influences stage switching. For alternative oxidase, immunostaining for bradyzoites and tachyzoite antigens is performed using gabaculine treated and control cultures. This is especially informative concerning whether bradyzoites utilize alternative oxidases exclusively, because gabaculine treatment of cultures would limit use of conventional oxidases and thereby select bradyzoites.

e) Western Blot Analysis, and ELISAs to determine stage specific expression of enzymes

Bradyzoites and tachyzoites also are compared directly for the relative amounts of alternative oxidase, using northern blot analyses, enzyme assays of parasites, isolation of mRNA and RT-PCR, using a competitor construct as an internal standard, and by Western blotting and ELISAs using antibodies to the enzymes (e.g., alternative oxidase). UDP-glucose-starch glycosyl transferase, chorismate synthase, isocitrate lyase, GSAT also are studied in a similar manner.

Example 8: Probing *Apicomplexan* DNA with Homologous Plant-like Genes or Potentially Homologous Genes From Other Parasites

The presence of the *gsa* genes, alternative oxidase genes, EPSP synthase genes, chorismate synthase genes, isocitrate lyase genes, and malate synthase genes are identified by probing, and then sequenced. For example, the cDNA clone of soybean *gsa* is labeled for chemiluminescent detection (ECL) or ^{32}P detection to identify homologous *gsa* sequences in *T. gondii*. Probes are used on a membrane containing the genomic DNA of *T. gondii* and soybean (positive control). When *T. gondii* genes are isolated, they are used to probe other Apicomplexan DNA. Thus, the *gsa* genes of *Cryptosporidia*, *Eimeria*, and *Malaria* are detected in the same manner as the *T. gondii* *gsa*.

In addition, DNA probes complementary to *Trypanosome* alternative oxidase DNA are used to probe the Apicomplexan DNA. The gene for *T. gondii* alternative oxidase is identified by screening *T. gondii* cDNA expression libraries using the 7D3 monoclonal antibody or the tobacco alternative oxidase gene used as a probe and thus detecting the gene expressing the relevant protein. This gene is used to detect the alternative oxidase genes of other Apicomplexan parasites by Southern analysis and screening other Apicomplexan cDNA libraries.

A nucleotide sequence generated from random sequencing of a *T. gondii* tachyzoite cDNA library and placed in the Genbank database was found to encode a protein with homology to tomato chorismate synthase. The EST was obtained, cloned and the full length sequence of the *T. gondii* chorismate synthase gene and deduced amino acid sequences were obtained (FIGS. 9 and 10). This provides evidence for these plant-like pathways and information useful in preparing a probe to isolate and

sequence this full gene from other Apicomplexan parasites as well. This gene was used as a probe and identified a chorismate synthase in *Eimeria bovis* DNA and *Cryptosporidium parvum* DNA. A *P. falciparum* EST has also been cloned and sequenced. Probes for *gsa* (soybean) alternative oxidase (soybean and tobacco), isocitrate lyase (cotton), UDP glucose starch glycosyl transferase (sweet corn), and acetohydroxy acid synthase (sweet corn) also are used to screen for clone, and sequence Apicomplexan genes. Large numbers of *T. gondii* genes from tachyzoite and bradyzoite cDNA libraries are being sequenced and deposited in Genbank. Putative homologous genes encoding plant enzymes are used to compare with these sequences to determine whether they are identified in the libraries and if so to determine whether the enzymes are encoded in the nucleus or plastid.

Example 9: __Identification of Genes Encoding Enzymes of the Plant-Like Biochemical Pathways in Apicomplexan__

Genes are isolated from a cDNA library by hybridization using specific probes to genes known to encode enzymes in metabolic pathways of plants. (see Example 9). Genes are cloned by complementation from a *T. gondii* cDNA expression library using a series of *E. coli* mutants that lack these enzymes and thus depend on the addition of exogenous additives for their optimal growth. Transformed bacteria are used to isolate and sequence plasmid DNA and from those sequences, probes are generated to determine whether other Apicomplexans have genes homologous to those in *T. gondii*

1) __cDNA libraries__ A cDNA library was constructed by Stratagene from mRNA isolated from *T. gondii* tachyzoites of the Me49 strain of *T. gondii* using the

Uni-ZAP XR cDNA library system. The titer of the amplified library is 1-2 X 10^{10}/ml. Other cDNA libraries also are utilized.

The phagemids were excised with R408 or VCS-M13 helper phage and transduced into XL1-Blue Cells. The plasmid DNA was purified using the Qiagen maxiprep system. Other libraries, *e.g.*, early Me49 bradyzoite, *in vivo* Me49 bradyzoite, and Me49 tachyzoite libraries also are suitable, as are other tachyzoite and bradyzoite libraries prepared by Stratagene.

2) Screening of library for genes. This is done in a standard manner using monoclonal or polyclonal antibodies or a radiolabeled gene probe.

3) cDNA expression libraries are probed with DNA from the genomes of:

a) *Toxoplasma gondii;*

b) *Plasmodium malariae;*

c) *Cryptosporidium parvum;*

d) *Eimeria.*

The existence of plant-like pathways is confirmed in members of the Apicomplexa by demonstrating the existence of genes encoding the enzymes required for the pathways. Genomic DNA is examined by Southern blot analysis for the presence of the sequences encoding enzymes required for specific algal or plant metabolic pathways. Genomic DNA is extracted from Apicomplexan parasites by proteinase K digestion and phenol extraction. DNA(5-10μg) is digested with restriction enzymes, electrophoresed through 1% Agarose and transferred to a nylon membrane. The ECL (Amersham) random prime system is used for labeling of DNA

probes, hybridization and chemiluminescence detection. Alternatively, the Boehringer Mannheim Random Prime DNA labeling kit is used to label the DNA with ^{32}P with unincorporated nucleotides removed using G-50 Sephadex Spin columns. Hybridization with the ^{32}P-labeled probe is carried out in [1M NaCl, 20 mM NaH_2 PO4 pH 7.0, 1% SDS, 40% formamide, 10% dextran sulfate, 5 mg/ml dry milk, 100 μg/ml salmon sperm DNA] at 37°C. Washes are optimized for maximum signal and minimum background. Probes are prepared from *T. gondii* cDNA clones obtained and characterized as described in Example 9. If lack of overall sequence conservation limits ability to detect homology, highly conserved regions are useful. For example, two highly conserved regions of the *gsa* gene are useful to generate oligonucleotide probes (Matters *et al.*, 1995).

4) PCR: An alternative approach for identifying genes encoding enzymes of the present invention is by using PCR with primers selected on the basis of homologies already demonstrated between plant protein sequences for the relevant gene. For example, for the *gsa* gene, polymerase chain reaction technology is used to amplify homologous sequences from a *T. gondii* cDNA library or *T. gondii* genomic DNA using primers generated from two highly conserved regions of GSAT. The *Neurospora crassa* alternative oxidase gene has been isolated using degenerate primers designed from conserved regions in alternative oxidase sequences from plant species (Li *et al.*, 1996). These primers are used to detect and clone the alternative oxidase gene from *T. gondii*. Candidate PCR products are cloned using the Invitrogen TA cloning kit

5) <u>Sequencing</u>: DNA from candidate cDNA clones is extracted using the Promega Wizard Miniprep System. Clones of interest are purified in large scale using the Maxiprep Protocol (Qiagen) and are sequenced by modified Sanger method with an automated sequencer (ABI Automated Sequencer) by the University of Chicago Cancer Research Center DNA Sequencing Facility.

6) <u>Homology Search</u>: to determine whether there is homology of isolated genes with other genes, *e.g. gsas*, sequences are compared against those in Genbank using the BLASTN (DNA → DNA) and BLASTX (DNA → Protein) programs. *T. gondii* sequence data is available in Genbank. Sequences for plasmodia also are available as are some isolated sequences for the other Apicomplexan parasites. *T. gondii* sequences are searched for homologies to the known plant genes *gsa*, glutamyl-tRNA reductase, isocitrate lyase, malate synthase, alternative oxidase, EPSP synthase, and chorismate lyase using the BLASTN (DNA→ DNA) and TBLASTN (Protein → Conceptual Translation of DNA Sequence) programs. The conserved plant gene sequences for the shikimate pathway are those described by Kahn *et al.* (1977) and Maloy *et al.* (1980; 1982). Conserved plant gene sequences for comparison of homologies are outlined by Klee *et al.* (1987). Similar libraries and sequence data for Plasmodia also are compared for homologies in the same manner.

7) <u>Complementation</u>: To isolate *T. gondii* genes or to demonstrate that a gene encodes a functional enzyme product, plasmids from the cDNA library detailed above, or modified constructs, are used to complement *E. coli* mutant strains GE1376 or GE1377 *(hemL)* and RP523 *(hemB)* from the Yale *E. coli* genetic stock center and

SASX41B (*hemA*) from D. Soll. This strategy has been successful for cloning *gsa* genes from plants and algae (Avissar and Beale, 1990; Elliott *et al.*, 1990; Grimm, 1990; Sangwan and O'Brian, 1993). The *hemA* gene encodes glutamate-tRNA reductase, an enzyme important in the C5-pathway for heme synthesis. The *hemB* gene encodes ALA dehydratase, an enzyme common to both heme biosynthesis pathways that should be common to all organisms and is included as a positive control. Mutant bacteria are made competent to take up DNA with $CaCl_2$ treatment and are transformed with plasmids from the cDNA library. Briefly, chilled bacteria (O.D. 550nm ~0.4-0.5) are centrifuged to a pellet and resuspended in ice-cold 0.1M $CaCl_2$ and incubated for 30 minutes on ice. Following further centrifugation, the cells are resuspended in 0.1M $CaCl_2$, 15% glycerol and frozen at -80°C in transformation-ready aliquots. 0.2ml ice-thawed competent bacteria are incubated on ice for 30 minutes with approximately 50ng plasmid DNA. Cells are placed at 43°C for 2.5 minutes and cooled on ice for 2 minutes. Following the addition of 0.8ml Luria Broth, cells are incubated at 37°C for 1 hour and 0.1ml is plated onto M9 minimal media plates. The M9 (Ausubel *et al.*, 1987) medium contains 0.2% glycerol as the carbon source, 1 mM $MgSO_4$, 0.1mM $CaCl_2$, 1 mM IPTG, 0.2 mg/ml Ampicillin, and 40 μg/ml threonine, leucine, and thiamine. Nonselective medium contains 25 μg/ml δ-aminolevulinic acid (*hemL* and *hemA*) or 4 μg/ml hemin (*hemB*). Alternatively, bacteria can take up DNA by electroporation. Chilled bacteria are prepared by a repetition of centrifugation and resuspension. The cells are washed in an equal volume of cold water, a ½ volume of cold water, a 1/50 volume of cold 10% glycerol, and finally in a

1/500 volume of cold 10% glycerol and frozen in 0.04 ml aliquots at -80°C. Cells are thawed at room temperature and chilled on ice. Cells are mixed with the DNA for 0.5-1 minutes and then pulsed at 25µF and 2.5 KV. The cells are rapidly mixed with SOC medium and grown at 37°C for 1 hour. Cells are plated in the same way as for $CaCl_2$ transformation.

Successful complementation of the *E. coli* mutants with a *T. gondii* gene is determined by plating the transformed bacteria onto minimal medium which lacks the supplement required for optimal growth of the *E. coli* mutant. Growth on the selective medium is compared to growth on nonselective medium, which contains 25 :g/ml δ-aminolevulinic acid (*hemL* or *hemA*) or 4 µg/ml hemin (*hemB*). Clones that complement each *E. coli* mutant are tested for their ability to complement each of the other mutants. Clones of putative *T. gondii gsa* and glutamate-tRNA reductase should complement only *hemL* and *hemA* mutants, respectively. Clones that suppress more than one *hem* mutation are candidates for alternative oxidase gene clones.

A cDNA clone containing the entire soybean *gsa* gene was able to transform the *E. coli hemL* mutant from auxotrophic to prototrophic for δ-aminolevulinic acid (ALA). Thus the system for obtaining *T. gondii* genes that complement *E. coli* mutants is available

For the glyoxylate cycle the mutants used for complementation are as follows: DV21 A01 (*aceA* which lacks isocitrate lyase) and DV21 A05 (*aceB* which lacks malate synthase)

For the shikimate pathway the mutants for complementation are available and used as follows: *E. coli, AroA* and yeast *AR*.

The same procedures are used for *Plasmodium falciparum* and *Plasmodium knowlesii, Cryptosporidium* and *Eimeria* complementation. When transit sequences lead to production of a protein which does not fold in such a manner that the protein can be expressed in *E. coli* or yeast constructs that lack these sequences are prepared to use for complementation that lack these sequences.

Example 10: <u>Analysis of Alternative Oxidases in *T. gondii*</u>

T. gondii bradyzoites use unique alternative oxidases. Alternative oxidases are necessary and sufficient for bradyzoite survival. Methods to characterize plant alternative oxidases are as described (Hill, 1976; Kumar and Söll, 1992; Lambers, 1994; Li *et al.*, 1996, McIntosh, 1994).

For *in vitro* studies, cell lines that lack functional mitochondria are used. These cell lines are used to allow the study of inhibitors effective against the conventional or alternative respiratory pathways within the parasite, but independent from their effects on the host cell mitochondria. SHAM, an inhibitor of the alternative respiratory pathway is used at concentrations between 0.25 and 2 μg/ml *in vitro*, and 200 mg/kg/day orally or parenterally *in vivo* alone or in conjunction with other inhibitory compounds. Other approaches include complementation of alternative oxidase-deficient *E. coli* mutants to isolate and sequence the alternative oxidase gene, immunostaining using antibodies for potentially homologous enzymes, enzymatic assay

and the creation of mutant-knockouts for the alternative oxidase gene and studying stage specific antigens in such knockouts.

1) Cell lines: Two cell lines, a human fibroblast cell line (143B/206) lacking mitochondrial DNA, and the parental strain (143B) which possess functional mitochondria are used. These cell lines have been demonstrated to support the growth of *T. gondii* (Tomavo and Boothroyd, 1995).

2) Inhibitor studies: Inhibitor studies are carried out as described herein. SHAM concentrations are 0.25 to 2 mg/ml *in vitro* and 200 mg/kg/day *in vivo*.

3) Immunostaining for tachyzoite and bradyzoites: Immunostaining is performed on cultures of fibroblasts in Labtech slides infected with tachyzoites (RH strain) as described herein. Slides are stained 1, 2, 4, 6 and 8 days post infection with anti-BAG and antiSAG1.

4) RT-PCR is as performed using the protocol of Hill (Chaudhuri et al., 1996) with degenerate primers based on consensus sequences. The product is cloned, sequenced and homology with known alternative oxidases documents its presence.

5) Complementation and alternative oxidase gene cloning: Complementation is used to demonstrate function and is an alternative approach to isolate the gene. Proper function of the complementation system is demonstrated by using complementation with a plant alternative oxidase gene. Mutants suitable for use are *hemL*, *hemA*, *hemB*. The alternative oxidase gene, AOX, is cloned from a *T. gondii* cDNA expression library by complementation of the *E. coli hemL* mutant. *HemL* mutants of *E. coli* cannot synthesize heme and are therefore deficient in respiration.

This cloning strategy has been successful in isolating AOX genes from *Arabidopsis* (Kumar and Soll, 1992). The procedure employed for recovering transformants is identical to that used for cloning the *T. gondii gsa* gene. The distinction between the *gsa* and AOX genes is that the AOX gene should restore function not only to *hemL* mutants but also to other *hem* mutants of *E. coli*. In addition, respiratory growth of *E. coli* on the alternative oxidase should be antimycin-insensitive and SHAM-sensitive. Clones recovered are tested for complementation of *hemL*, *hemB* and *hemA* mutants. Growth is tested for inhibitor sensitivity. Sequences of cDNA clones that provide functional alternative oxidase activity by these tests are compared with known AOX gene sequences (McIntosh, 1994).

The *Escherichia coli* strain XL1-Blue was prepared for infection with the *T. gondii* phage library according to Stratagene manufacturer's protocol. The RH tachyzoite library in the λ-ZAP vector system was titred, and 10^6 pfu are added to the XL1-Blue preparation. Approximately 6 X 10^5 plaques are plated on agar onto 150 mm^2 petri dishes containing NZY medium, and grown at 42°C for 3.5 or 8 hours, depending upon which screening method is employed. If antibodies are used for screening, IPTG-soaked nitrocellulose filters are placed on the plates after the short incubation period, and the growth of the plaques is allowed to proceed for an equivalent period of time. Filters are blocked in BLOTTO overnight. Screening is carried out under the same conditions which had been optimized during Western blotting with that primary antibody, and the appropriate secondary antibody. If DNA probes are used for screening, the plaques are grown for 8 hours post-infection, and

placed at 45°C for 2 hours to overnight. Nitrocellulose filters are placed on the plates, and all subsequent steps for lysis and fixing of the DNA are as specified in the Stratagene protocol. Filters are placed into a pre-hybridization solution containing Denhardts, SSC, SDS, and denatured salmon sperm DNA, as directed in Ausubel *et al.* (1987). Blots are hybridized to ^{32}P-labeled probe overnight. Low stringency washes, containing 5X SSC and 0.1% SDS are performed twice at room temperature, and high stringency washes with 0.2X SSC and 0.1% SDS are performed at a temperature dependent upon the degree of homology between the probe and the *T. gondii* DNA.

6) <u>Assays for the presence of genes</u>: Evidence for the presence of the genes which encode the novel enzymes is obtained by demonstrating enzyme activity and/or Western blot analysis of Apicomplexan whole cell lysates and/or polymerase chain reaction and/or probing the genomic DNA of the parasite with the homologous DNA. Identification of the genes is accomplished by screening an Apicomplexan cDNA library with the antibody to homologous enzymes from plants or other microorganisms or probes which recognize the genes which encode them and/or complementation of mutant bacteria lacking the enzyme with Apicomplexan DNA.

7) <u>Mutant-Knockouts</u>: The alternative mitochondrial oxidase pathway is the preferred oxidative pathway for bradyzoites and is likely to be important for their survival. The genetic system used to examine the function of the gene via targeted gene knock-outs and allelic replacements essentially as described (Donald & Roos, 1993, 1994, 1995). The alternative oxidase is not absolutely required for growth when cytochrome oxidase can be active and mutants are recoverable. The AOX-null strains

may be hypersensitive to GSAT inhibitors, both *in vitro* and *in vivo*. The ability of the AOX-null strains to switch stages, both *in vitro* and *in vivo* is determined. The AOX-null strains are examined for stage specific antigens. Virulence and ability to form cysts are assessed *in vivo* in C3H/HeJ mice as described herein.

Knockouts with a bradyzoite antigen reporter gene are produced and these constructs and organisms with the genes knocked out are cultured under conditions that would ordinarily yield a bradyzoite phenotype. These are used to determine whether expression of the "knocked out" gene is critical for bradyzoite antigen expression and the bradyzoite phenotype.

8) Similar "knockouts" of EPSP synthase or chorismate synthase are produced.

9) Similar procedures are used for other Apicomplexan parasites. For example, a similar genetic system is available for *P. falciparum*.

Example 11: Production, Testing, and Use of Vaccines against Apicomplexa

"Knock out" organisms (*e.g.*, lacking GSAT, or alternative oxidase or EPSP-synthase or chorismate synthase or UDP-glucose starch glycosyl transferase) are produced as described herein. The knock-out vaccine strain in some cases is cultivated in tissue culture because components which are deficient are provided by a single product or a plurality of products. DNA constructs and proteins are produced and tested as described herein (see Materials and Methods) using unique genes and sequences and assay systems and methods which are known to those of skill in the art and disclosed herein. Briefly, they are used to immunize C3H mice, and tissues of

immunized and control mice are subsequently examined for persistence of parasites. These immunized mice and controls are challenged perorally with 100 cysts of Me49 strain or intraperitoneally with 500 RH strain tachyzoites. Effect of immunizations on survival, and tissue parasite burden are determined (McLeod *et al.*, 1988). Parasite burden refers to quantitation of numbers of parasites using PCR for the B1 *T. gondii* gene, quantitating numbers of cysts in brain tissue, quantitating numbers of parasites by inoculating serial dilutions of tissues into uninfected mice when the RH strain of *T. gondii* is utilized and assessing survival of recipient mice as 1 parasite of the RH strain of *T. gondii* is lethal. Ability to prevent congenital transmission and to treat congenital infections is also a measure of vaccine efficacy. Vaccines are useful to prevent infections of livestock animals and humans. Standard methods of vaccine development are used when substantial prevention of infection is achieved in murine models.

Example 12: Nucleotide and Deduced Amino Acid Sequence of *T. gondii* Chorismate Synthase cDNA

Animals and most protista (*e.g. Leishmania*) rely exclusively on exogenous folates. Previous studies which demonstrate the efficacy of anti-folates for the treatment of toxoplasmosis have implied that *T. gondii* has the enzymes necessary to synthesize folates. For this purpose, *T. gondii* uses PABA. The biochemical events that lead to PABA production in *T. gondii* or any other Apicomplexan have not been previously characterized. In algae, plants, certain bacteria and fungi, the shikimate pathway facilitates the conversion of shikimate to chorismate, a three step reaction

catalyzed by three enzymes, shikimate kinase, 3-phospho-5-enolpyruvyl shikimate synthase (EPSP synthase) and chorismate synthase. Chorismate is then used as a substrate for the synthesis of PABA. In plants, EPSP-synthase and chorismate synthase are encoded in the nucleus. In plants, algae and bacteria, chorismate is not only an essential substrate for the synthesis of folate, but it is required for the synthesis of ubiquinone and certain aromatic amino acids. The shikimate pathway may occur both inside and outside of the plastid: For example, EPSP synthase exists in two forms in *Euglena*, one associated with the plastid of those grown in the light and the other found in the cytosol of those grown in the dark.

Apicomplexan parasites utilize the shikimate pathway for folate synthesis. An inhibitor of the EPSP synthase, an essential enzyme in this pathway, restricts the growth of *T. gondii*, *P. falciparum* and *C. parvum in vitro*. This inhibitor, NPMG, synergizes with pyrimethamine and sulfadiazine to prevent *T. gondii* multiplication. NPMG also synergizes with pyrimethamine to protect mice against challenge with the virulent RH strain of *T. gondii*. The sequence of a *T. gondii* gene that encodes a putative chorismate synthase, that has considerable homology with chorismate synthases from other organisms, provides information useful in developing novel antimicrobial agents.

A partial cDNA sequence of approximately 250 bases was identified from the "Toxoplasma EST Project at Washington University." This sequence, when translated, had approximately 30% homology with chorismate synthase from a number of organisms. Both strands of the corresponding clone were sequenced and found to be 2312 bases in length (FIG. 9). Analysis revealed a large open reading frame of 1608

base pairs which would encode a 536 amino acid protein. Homology was determined by the use of CLUSTAL X, a computer program that provides a new window base user interface to the CLUSTAL W multiple alignment program. (Thompson, 1994). The deduced amino acid sequence has considerable identity (44.5 to 51.4%) with chorismate synthases of diverse species (FIG. 10). The putative *T. gondii* protein differs from other known chorismate synthases in length. Chorismate synthases from other organisms range in length from 357-432 amino acids. The larger size of the *T. gondii* protein is due to an internal region that has no counterpart in other known chorismate synthases and is novel. The function of this region remains to be determined. The *T. gondii* chorismate synthase sequence was used in a search with the BLAST program. An EST from a *Plasmodium falciparum* cDNA library was located that has considerable homology with the *T. gondii* sequence. Chorismate synthase is also present in *Mycobacterium tuberculosis*.

The nucleotide sequence of the cDNA which encodes a putative *T. gondii* chorismate synthase and the amino acid sequence deduced from it is shown in FIG. 9. The deduced amino acid sequence of putative *T. gondii* chorismate synthase has substantial homologies with chorismate synthases from diverse organisms including *Solanum lycospersicum* (tomato), *Synechocystis species*, *Hemophilus influenza*, *Saccharomyces cerevisiae*, and *Neurospora crassa*. (FIG. 10).

The Apicomplexan data base in Genbank was searched for homologies to the *T. gondii* chorismate synthase gene. A homologous *P. falciparum* EST (FIG. 11) was

identified. It was sequenced. This provided additional evidence that at least a component of the shikimate pathway also was present in *P. falciparum*.

Sequencing Method

Characterization of Insert and Design of Sequencing Strategy.

Clone TgESTzy11c05.r1 was obtained from the Toxoplasma project at Washington University and supplied in the Bluescript SK vector as a phage stock. Phagemid DNA was excised by simultaneously infecting XL1-Blue cells with the phage stock and VCS-M13 helper phage. Purified phagemids were used to infect XL1-blue cells. Infected XL1-Blue cells were grown in LB media and plasmid DNA purified using Qiagen maxi-prep kits. The cDNA insert was excised using EcoR I and Xho I restriction enzymes and found to be approximately 2.4KB. Initial sequencing of the 5 prime end of the insert's plus strand and its translation, revealed 30% homology with previously described chorismate synthases from other organisms. However, sequencing of the 5 prime end of the minus strand yielded a sequence that when translated had little apparent homology with any known protein. A series of restriction digestion experiments were performed to establish a restriction map of the insert. Restriction fragments were electrophoresed through a 1% agarose gel and fragments visualized by ethidium bromide staining and ultra-violet illumination. Due to the lack of available restriction enzyme sites within the insert, sequencing with the conventional technique of using sub-cloned overlapping restriction fragments as templates would prove to be laborious and time consuming. To circumvent this potential problem and facilitate rapid sequencing, a strategy was designed that used both conventional sub-cloned

overlapping restriction fragments with standard vector annealing primers and the full length clone with custom designed primers. Thus, sequencing was first carried out by using sub-cloned restriction fragments and the information obtained used to custom design unique sequencing primers. These primers allowed efficient sequencing of the internal regions and the external 3 prime end of each strand. The customized primers were:

<u>CUSTOMIZED PRIMERS</u>:

CS1 5' TGT CCA AGA TGT TCA GCC T 3'

CS2 5' AGG CTG ATC ATC TTG GAC A 3'

CS2 5' TCG GGT CTG GTT GAT TTT 3'

CS4 5' GAG AGA GCG TCG TGT TCA T 3'

CS5 5' ATG AAC ACG ACG CTC TCT C 3'

CS6 5' CAT GTC GAG AAG TTG TTC 3'

CS7 5' GAA CAA CTT CTC GAC ATG 3'

CS8 5' ACT TGT GCA TAC GGG GTA C 3'

CS9 5' GTA CCC CGT ATG CAC AAG T 3'

CS10 5' TGA ATG CAA CTG AAC TGC 3'

CS11 5' GCA GTT CAG TTG CAT TCA 3'

CS12 5' AGC CGT TGG GTG TAT AAT C 3'

CS13 5' CTA CGG CAC CAG CTT CAC 3'

CS14 5' CGT CCT TCC TCA ACA CAG TG 3'

CS15 5' GTG AAG CTG GTG CCG TAG 3'

CS16 5' CGC CTC TGA TTT GGA AGT G 3'

CS17 5' TCT GCC GCA TTC CAC TAG 3'

CS18 5' GAA GCC AAG CAG TTC AGT T 3'

Sub-cloning

Sub-clones were made from restriction fragments isolated by agarose gel electrophoresis and purified using the Qiaex gel extraction kit Qiagen, Chatsworth CA. Double digestions of the plasmid with Hinc II and Pst I resulted in 4 fragments of 500, 800, 300 and 4000 base pairs. The 800 bp fragment, corresponding to the base pairs 800-1600 was ligated into the bluescript KS vector. The 1600-2400 base pair portion of the insert was obtained in a similar manner using Pst I and Xho I restriction enzymes and ligated into the bluescript KS vector. Ligations were performed for 12 hours at 18 degrees centigrade on a PTC-100, programmable thermal cycler, MJ Research Inc. Watertown, Massachusetts. Plasmids containing the restriction fragments were used to transform DH5α competent cells. Plasmid DNA was purified using Qiagen maxi-prep kits.

Primer Sequence Design

Primers were designed based on the sequencing information obtained from restriction enzyme fragments. To facilitate sequencing of a region on the same strand and 5 prime to an already sequenced portion of insert, primers were designed from an area approximately 200-300 nucleotides 5 prime into the last obtained sequence. For sequencing of the complementary strand, primers were designed to be the complement

and reverse of the same region. Primers were designed to be 18-25 nucleotides in length and have a Tm of 55-60 degrees centigrade. G plus C content was 45-55 percent. Primers were designed to have minimal self annealing and to have a low propensity for primer to primer annealing. Primers with the ability to form stable secondary structures were not designed. These criteria for the design of primers were based on theoretical considerations and results of other experiments which found that primers which had Tms of much less than 55 degrees centigrade failed to work or performed poorly, producing ambiguous sequences of low quality.

Sequencing and Assembly of Sequence Information.

All sequencing was performed using a Perkin Elmer automated sequencer. The three purified plasmids containing the entire cDNA or a restriction fragment were used as templates for sequencing reactions with the standard M13 and reverse primers. The sequences obtained were used to design primers which allowed sequencing of the internal regions of the inserts. This process was repeated until both strands of the entire clone were sequenced. Chromatograms were critically edited and controlled for quality using Sequencher software. Edited chromatograms of excellent quality were assembled with the same software package and a consensus sequence obtained. The consensus sequence was analyzed for open reading frames using Macvector software package. Kodak International Biotechnology, Inc., New Haven, CT.

Example 13: Transit Sequence of *T. gondii* Chorismate Synthase

Homology with other peptides was sought using the Genbank database and the unique sequence in the *T. gondii* chorismate synthase (amino acids 284 to 435,

Figure 11) There was thirty percent identity and forty-five percent homology, with a number of conserved motifs, between this unique sequence of *T. gondii* chorismate synthase and the amyloplast/chloroplast transit (translocation) sequence of the Waxy protein (UDP-glucose starch glycosyl transferase) of *Zea mays* (sweet corn). The same methods whereby the *Zea mays* transit sequence was analyzed (Klosgen and Well, 1991), i.e., construction of the transit sequence with a reporter protein, immunolocalization of the protein, creation of the construct with deletions or mutations of the transit sequence and subcellular immunolocalization using immunoelectronmicroscopy are useful for proving that this is a transit sequence in the *T. gondii* chorismate synthase. A useful reporter protein for a chimeric construct is β glucoronidase of *E. coli*, expressed under the control of the 35S promoter of cauliflower mosaic virus. The β glucoronidase alone is expressed, in parallel. The transit peptide chimeric construct is found in the plastid. The control β glucoronidase is found in the cytoplasm. Another useful reporter system is green fluorescent protein (gfp). Antibodies to the chorismate synthase protein are also used to detect the presence of the product of the gene (with the transit sequence) in the plastid and the product of a construct in which the transit sequence is not present in the cytoplasm only. This is used to immunolocalize proteins in different life-cycle stages. Further mutations and deletions are made which identify the minimal transit sequence using the same techniques as described above for the entire peptide. Antisense, ribozyme or intracellular antibodies directed against the transit sequence nucleic acid or translated protein are useful as medicines. The amino acid or nucleic acid which encodes the

transit sequence are the bases for diagnostic reagents and vaccine development. This transit sequence is useful for the construction of ribozyme, antisense nucleic acids, intracellular antibodies which target a key parasite protein, and creation of constructs with accompanying molecules which are lethal to the parasites (Roush, 1997; Mahal *et al.*, 1997). This transit sequence also is useful because it provides a general extension of the concept of transit and targeting sequences in Apicomplexan parasites that enable targeting of other parasite organelles in addition to plastids. The transit sequence of *Zea mays* and *T. gondii* are shown in Figure 11.

Example 14. <u>Nucleotide and Deduced Amino Acid Sequences of *P. falciparum* Chorismate Synthase EST.</u>

Sequencing of *P. falciparum* chorismate synthase EST followed the same pattern as described above for sequencing the *T. gondii* chorismate synthase gene with the following exceptions: There was difficulty in obtaining sequence from the 3' region of the cDNA due to an unstable polyA tail. This made it necessary to do all sequencing approaching from the 5' end using gene walking techniques and subcloning of restriction fragments. The AT richness of *P. falciparum* genes increased the complexity of design of the customized primers. The customized primers utilized were:

PFCS1 AGC TAT TGG GTG GATC

PFCS2 TCC ATG TCC TGG TCT AGG

PFCS3 ATA AAA ACA CAT TGA CTA TTC CTT C

PFCS4 GGG GAT TTT TAT TTT CCA ATT CTT TG

PFCS5 TTG AAT CGT TGA ATG ATA AGA C

PFCS6 TTT TAG ATC AGC AAT CAA ACC

PFCS7 AAC TTT TTA TCT CCA TAC TTT G

PFCS8 GAA GGA ATA GTC AAT GTG TTT TTA T

PCFS9 GTA TTT TAC CAA GAT TAC CAC CC

PFCS10 CCC CCA ACA CTA TGT CG

PFCS11 CAG TGG GCA AAA TAA AGA

PFCS12 CCA GTG GGC AAA ATA A

PFCS13 GGA AGA GAA ACA GCC AC

PFCS14 TGC TGC TGG GGC GTG

The gene and deduced amino acid sequences are in Figure 12.

Example 15: Southern Blotting Demonstrates Presence of Chorismate Synthase (and by Inference all of the Shikimate Pathway) in Apicomplexan Parasites

Southern blotting using the *T. gondii* chorismate synthase gene as a ^{32}P labeled probe demonstrated homology at moderate stringency (e.g. 0.2 x SSC, 0.1% SDS at 42°C) [more stringent conditions define greatest relatedness of genes] with *Eimeria bovis* and *Cryptosporidium parvum* DNA.

This *T. gondii* cDNA also comprises a probe for screening cDNA libraries of all other Apicomplexa to identify their chorismate synthase genes The same principles are applicable to all the other enzymes in Table 1

Example 16: <u>Gene Expression. Recombinant Protein. Production of Antibody and Solving the *T. gondii* and *P. falciparum* Crystal Structures of chorismate synthase to establish their active site and secondary structure.</u>

These are done using standard techniques. The gene construct is placed within a competent *E. coli*. Recombinant enzyme is identified by homologous antibody reactivity and purified using affinity chromatography. Fusion proteins are useful for isolation of recombinant protein. Protein is injected into rabbits and antibody specific to the protein is obtained and utilized to purify larger amounts of native protein for a crystal structure. The crystal structure provides information about enzyme active site and facilitates rational drug design (Craig and Eakin, 1997). Recombinant proteins are used for high through put screens to identify new antimicrobial agents.

Example 17: <u>Other Uses (e.g. in diagnostic reagents and vaccines) of the Chorismate Synthase Gene as a Representative Example of Uses of Each of the Genes and Enzymes in These Pathways That are not Present or Rarely Present in Animals.</u>

These uses include *T. gondii* genes and proteins used as diagnostic reagents and as a vaccine to protect against congenital infection. Recombinant protein (all or part of the enzyme) is produced and is used to elicit monoclonal antibodies in mice and polyclonal antibodies in rabbits. These antibodies and recombinant protein (e.g., to *T-gondii* chorismate synthase) are used in ELISA (e.g. antibody to human IgG or IgM, or IgA or IgE attached to ELISA plate + serum to be tested + antibody conjugated to

enzyme ÷ enzyme substrate). The recombinant proteins, pooled human sera from known uninfected individuals (5 individual sera pooled) and infected individuals (5 individuals with acute infection sera pooled, 5 individuals with chronic infection sera pooled) are the controls. This test is useful with serum or serum on filter paper.

Another example of a diagnostic reagent are primers to amplify the target transit sequence or another portion of the chorismate synthase sequence unique to *T. gondii*. PCR with these primers is used with whole blood to detect presence of the parasite. Such assays have proven to be useful using the *T. gondii* B1gene (Kirisits, Mui, Mack, McLeod, 1996).

Another example of a diagnostic reagent is useful in outpatient settings such as an obstetrician's office or in underdeveloped areas of the world where malaria is prevalent. FABs of monoclonal antibodies (which agglutinate human red cells when ligated) (Kemp, 1988) are conjugated to antibodies to the target sequence or selected enzyme. Antigen conjugated anti-red cell Fab also is used to detect antibody to the component. A positive test occurs when the enzyme or antibody is circulating in the patient blood and is defined by agglutination of red cells (in peripheral blood from the patient) mixed with the conjugated antibodies. Controls are the same as those specified for the ELISA.

Examples of vaccines are protein, peptides, DNA encoding peptides or proteins These are administered alone or in conjunction with adjuvants, such as ISCOMS. These vaccine preparations are tested first in mice then primates then in clinical trials Endpoints are induction of protective immune responses, protection measured as

enhanced survival, reduced parasite burden, and absent or substantial reduction in incidence of congenital infection (McLeod et al., 1988).

Example 18: *T. gondii* Chorismate Synthase Genomic Sequence

Genomic clones are isolated from commercially available genomic libraries (AIDS repository) using the identified cDNA clones as probes in the screening process. The genomic library, as λ phage, is isolated onto NZY agar plates using XL1-Blue *E. coli* as the host, resulting in plaques following a 37°C incubation. The cDNA sequence is radiolabeled with ^{32}P and hybridized to nylon membranes to which DNA from the plaques has been covalently bound. Plasmids from candidates are excised and their restriction enzyme-digested inserts sequenced. Experimental details are as described in Ausubel *et al.* (1987).

Example 19: *P. falciparum* Chorismate Synthase Genomic Sequence.

This is done with a gene specific subgenomic library as described in Example 18 (see example 41).

Other examples of enzymes and the genes which encode them and which are characterized as outlined above include: glutamyl-tRNA-synthetase; glutamyl-tRNA reductase; prephenate dehydrogenase aromatic acid aminotransferase (aromatic transaminase); cyclohexadienyl dehydrogenase tryptophan synthase alpha subunit; tryptophan synthase beta subunit; tryptophan synthase beta subunit; indole-3-glycerol phosphate synthase (anthranilateisomerase), (indoleglycerol phosphate synthase), anthranilate posphoribosyltransferase; anthranilate synthase component I; phosphobiosyl anthranilate isomerase anthranilate synthase component II; prephenate dehdryatase

(phenol 2-monooxygenase) catechol 1,2-deoxygenase (phenol hydroxylase); cyclohexadienyl dehydratase; 4-hydroxybenzoate octaprenyltransferase; 3-oxtaprenyl-4-hydroxybenzoate carboxylyase dehydroquinate synthase (5-dehydroquinate hydrolase); chorismate synthase (5-enolpyruvylshikimate 3-phosphate phosph-lyase); dehydroquinate dehydratase; shikimate dehydrogenase; 3-deoxy-d-arabino-heptuloonate 7 phosphate synthase; chorismate mutase (7-phospho-2-dehydro-3-deoxy-arabino-heptulate aldolase); 3-deoxy-d-arabino-heptuloonate 7 phosphate synthase; shikimate 3-phosphotransferase (shikimate kinase); UDP glucose starch glycosyl transferase; Q enzymes; acetohydroxy acid synthase; chorismate synthase malate synthase, isocitrate lyase; 3-enolpyruvylshikimate phosphate synthase (3-phosphoshikimate-1 carboxyvinyltransferase).

Example 20: *T. gondii* Chorismate Synthase, EPSP Synthase, and Shikimate Kinase Activities were Demonstrated

Assay for chorismate synthase, EPSP synthase and shikimate kinase in *T. gondii* were performed and demonstrated such activity.

Example 21: *T. gondii* Dehydroquinate Dehydratase Activity is Demonstrated

An assay for dehydroquinate dehydratase in *T. gondii* was performed and demonstrated such activity.

Example 22: GSAT activity is demonstrated in *T. gondii* tachyzoite lysates

An enzymatic assay (Sangwan and O'Brian, 1993) demonstrates GSAT activity in *T. gondii* lysates. The buffer contains 0.1 M MOPS (3-[N-morpholino]propanesulfonic acid), pH 6.8, 0.3M glycerol, 15 mM $MgCl_2$, 1 mM

dithiothreitol, 20 μM pyridoxal phosphate, 1 mM PMSF (phenylmethylsulfonyl fluoride). The MOPS, glycerol and $MgCl_2$ are combined and then pH'd. This is important because the glycerol alters the pH, so it must be added first. This is filter sterilized and has a long shelf life. When the buffer is needed, DTT, pyridoxal phosphate and PMSF are added immediately prior to use. The protein extract stock should be ~10 mg/ml if possible. The principle of the assay is conversion of substrate which produces a change in color due to the reactant.

Example-23: Isocitrate lyase activity is demonstrated in *T. gondii* tachyzoite lysates

An enzymatic assay demonstrates isocitrate lyase activity in *T. gondii* isolates prepared by disruption of the parasite membranes using french press or a lysis buffer. Demonstration that the lysis buffer does not alter enzyme activity is carried out by performing the assay with known substrate and enzyme in the lysis buffer and documenting presence of enzyme activity.

Example 24: Alternative oxidase activity is demonstrated in *T. gondii* preparations.

T. gondii tachyzoites and bradyzoites are assayed for alternative oxidase activity and such activity is found to be present in greater amounts in bradyzoites.

Example 25: Novel Substrate Competitors and Transition State Analogues of Enzymes Inhibit Apicomplexan Enzymes

Some inhibitors are competitive substrates or transition state analogues and they are utilized in the enzyme assay, *in vitro* with tachyzoite and bradyzoite

preparations and with native enzyme, tissues culture assays and in *in vivo* models as described above. These provide a model paradigm for designing inhibitors of any of the enzymes specified above. Briefly, inhibitors are produced as follows: Competitive substrates are produced by designing and synthesizing compounds similar to known compounds but modified very slightly. For example, inhibitors related to glyphosate are known. The structures of glyphosate, sulfosate and the precursor for EPSP have similarities (please see below). Inhibitors are designed by modifying substrates in such a manner that the modification interferes with the enzyme active site. This can be performed using molecular modeling software. Similarly, halogenated substrates for other enzymes have functioned effectively as nontoxic inhibitors. The principles are applicable to the design of inhibitors for any of the unique enzymes with well characterized substrates and active sites.

The approaches to rational design of inhibitors include those standard in the art (Craig and Eakin, 1997; Ott *et al.*, 1996). These methods use information about substrate preference and three-dimensional structure of the target enzyme (e.g., chorismate synthase or EPSP synthase).

In one approach, the structure of the target is modeled using the three-dimensional coordinates for amino acids in a related enzyme. An example of this is that the crystal structure of GSAT from a plant has been solved and its active site is known.

In another part of this approach, expression of high levels of recombinant enzyme is produced using cDNA (e.g., the chorismate synthase of *T. gondii* or *P.*

falciparum) and quantities of protein adequate for structural analysis, via either NMR or X-ray crystallography are obtained.

Drug resistant mutants are produced *in vitro* following mutation with nitrosoguanidine and culture with the inhibitor. The surviving organisms have acquired resistance to the inhibitor. This process is carried out either with the Apicomplexan parasite or with bacteria or yeast complemented with the gene encoding the enzyme or part of the gene (e.g., without the transit sequence). PCR amplifies the relevant cDNA and this cDNA encoding the resistant enzyme is cloned and sequenced. The sequence is compared with that of the enzyme that is not resistant. With the information about the inhibitor target and three-dimensional structure, the point mutations which cause resistance are analyzed with computer graphic display. This information provides the mechanism for altered binding of the drug, and the inhibitory compound is then modified to produce second generation medicines designed to treat resistant pathogens prior to their development in nature.

An example of the use of toxic analogues to kill parasites used by others provides a means whereby there is production of analogues toxic to parasites. Specifically, the purine analogue prodrugs, 6 sulfanylpurinol, 6 thioguanine, 6 thioxanthine and allopurinol interact with hypoxanthine phosphoribosyltransferase which is responsible for salvage of purines used to produce AMP and GMP. Such toxic analogues are effective against the plant-like enzymes in the pathways (see Table 1) in Apicomplexans.

Transit state analogues bind with extraordinarily high efficiency to the enzyme active site and are predicted from the three-dimensional structure and kinetic information. Analogues that mimic the structural properties and electrostatic surface potentials for the transition state are designed and synthesized. Empirical testing using recombinant enzyme demonstrates that these transition state analogues are good leads with high affinity for the active site of the target enzyme.

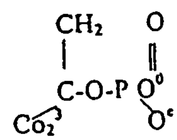
Multisubstrate analogues are useful because they markedly enhance the binding affinity to the enzyme. Similarly, if enzymes in a cascade are linked in such a manner that the substrate for one reaction provides the substrate for the next reaction, multisubstrate analogues are more useful.

Selective inhibitor design and lead refinement: Co-crystallization of inhibitors with target enzymes of host and pathogen enable three-dimensional analysis of molecular constructs and atomic interactions between inhibitors and enzymes and redesign of inhibitors (leads) to enhance their affinity for the pathogen enzyme. Iterative crystallography, lead redesign and inhibitor testing *in vitro* and *in vivo* enable design and development of potent selective inhibitors of the target of the pathogen enzyme. Recombinant methods for screening large numbers of analogues for those that bind selectively to the enzymes of specific parasites provide justification for inclusion of the analogues which bind best in the design of transition-state or multisubstrate analogues.

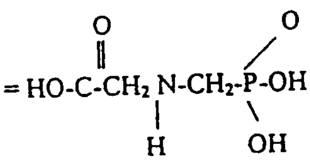
Additional examples (included to illustrate principles employed) but already patented by others include **Inhibitor of EPSP synthase** have been designed based on the

similarities of the inhibitor to the substrate. Based on molecular modeling algorithms additional inhibitors are designed.

Phosphoenolpyruvate =



glyphosate =



Inhibitors that effect components of these pathways are halogenated substrates or analogues which are effective competitors.

Inhibitors of Ubiquinone: Modifications (substitutions) of benzhydroxamic acids produce CoQ (ubiquinone) analogues such as esters of 2, 3 and 3,4 dihydroxybenzoic acid and structurally related compounds.

Inhibitor of Isoleucine/valine biosynthetic pathway: These are noncompetitive inhibitors as is shown by the lack of relatedness of the inhibitors (e.g., imidazolinones, sulfonylureas) to the target enzymes.

Inhibitors of GSAT

The following acids (5 amino-1.3 cyclohendienyl carboxylic acid, 4 amino 5 hexynoic acid (acetylenic, GABA), 4 amino 5 hexonoic acid (vinyl GABA) 2 amino 3 butanoic acid (vinyl glycine), 2 amino 4 methoxy-trans-3 butenoic acid. 4 amino 5 fluoropentanoic acid alter catalysis dependent formation of a stable covalent adduct

Inhibitors of lysine biosynthetic pathway: There are noncompetetive inhibitors of lysine synthesis that target enzymes in this patway (e.g., azi DAP, 3, 4 didehydro DAP, 4 methylene DAP4, 4 methylene DAP6) and inhibitors of other plant-like enzymes as in the Table 1A and B.

Example 26: Modifications of Inhibitory Compounds to Improve Oral Absorption Tissue Distribution (especially to brain and eye).

Tissue distribution is characterized using radiolabeled inhibitor administered to mice with its disposition to tissues measured by quantitation of radiolabel in tissues. Compounds are modified to improve oral absorption and tissue distribution by standard methods.

Example 27. Efficacy of Antimicrobial Compounds Alone. Together and In Conjoint Infections in Murine Models:

Inhibitors of plant-like pathways are effective against the Apicomplexan infection alone, together with the bacterial and/or fungal infections and also treat the bacterial and fungal infections alone.

Presence of inhibitory activity of new antimicrobial compounds is tested using Apicomplexans, bacteria and fungi in enzymatic assays, *in vitro*, and *in vivo* assays as described above and known to those of skill in the art.

Infections are established in murine models and the influence of an inhibitor or combination of inhibitors on outcomes are determined as follows:

Infections: Infections with *Toxoplasma gondii*, *Pneumocystis carinii*, *Mycobacterium tuberculosis*, *Mycobacterium avium* intracellular and *Cryptosporidium parvum* are established alone and together using an immunosuppressed rodent model. Endpoints in these infections are:

Survival: Ability of an inhibitor to protect the infected animal is measured as prolonged survival relative to the survival of untreated animals.

Parasitemia: Is a measure using isolation of mRNA and RT-PCR. A competitive inhibitor is used for quantitation.

Tissue Parasite Burden: Is determined by quantitating brain and eye cyst numbers.

Inflammatory Response: This is noted in histopathologic preparations.

Representative combinations of inhibitors are NPMG and sulfadiazine, SHAM and atovaquone, NPMG and pyrimethamine, NPMG and SHAM.

Example 28. Establishing Efficacy, Safety, Pharmakokinetics, and Therapeutic/Toxic Index:

The testing in murine models includes standard Thompson tests. Testing of antimicrobial agents for efficacy and safety in primate models for malaria is performed Dosages are selected based on safety information available from data bases of information concerning herbicides and the literature. Measurements of serum and tissue levels of antimicrobial compounds are performed using assays which detect inhibitor concentrations and concentrations of their metabolites Representative assays are high performance liquid chromatography, and assaying tissues for percentage of

radiolabeled compounds administered, using liquid scintillation, and other assays also are used.

Example 29. Determining whether there is Carcinogenicity and Teratogenicity:

Standard assays to evaluate carcinogenicity and teratogenicity include administration of medicines as described above to rodents and observation of offspring for teratogenic effects and carcinogenicity (i.e. development of malignancies). Observation includes general physical examination, autopsy and histopathologic studies which detect any teratogenic or carcinogenic effects of medicines.

Example 30. Constructs to Measure Parasitemia:

Portions of genes are deleted and the shorter gene is used as an internal standard in RT PCR assays to measure amount of parasites present (Kirisits, Mui, McLeod, 1996).

Example 31. Vaccine Constructs and Proteins and their Administration:

These are prepared, as described. They include DNA constructs (Ulmer, Donnelly and Liu, 1996) with the appropriate gene or portions of the gene alone or together, with adjuvants. Representative adjuvants include ISCOMS, nonionicsurfactant, vesicles, cytokine genes in the constructs and other commonly used adjuvants. Native and recombinant proteins also are used in studies of vaccines. Protection is measured using immunologic *in vitro* assays, and assessing enhanced survival, reduction of parasitemia tissue and parasite burden and prevention of congenital infection [McLeod et al., 1988]

Example 32: Stage-Specific Expression of Proteins

This is evaluated by enzyme assays, northern or western analysis, ELISA, semi-quantitation of mRNA using RT-PCR with a competitor as internal standard in gene-knockout organisms using culture conditions (e.g. alkaline pH, increased temperature, nitric oxide exposure) which ordinarily elicit a bradyzoite phenotype, or engineering a reporter construct and characterizing presence of the reporter in stage specific expression of antigens. Ability to change between life cycle stages or to persist in a particular life cycle stage is affected by presence or absence of particular plant-like genes and by treatment of inhibitors with plant-like processes. Suitable examples of plant-like enzymes which make parasites less able to switch from or persist in a specific life cycle stage include: alternative oxidase, enzymes critical for amylopectin synthesis such as starch synthases, UDP glucose-glucosyl starch transferase and branching (Q) enzymes.

Example 33. Preparation of Diagnostic Test Reagents and Diagnostic Tests:

These assays are as described (Boyer and McLeod, 1996). Sensitivity and specificity are established as is standard in the field. Tests and reagents include ELISAs in which antibodies to the proteins or peptides and recombinant proteins of this invention such as chorismate synthase (*Aroc)* are used and PCR methodology in which primers to amplify DNA which encodes the enzymes, or parts of this DNA, are used. A test useful in an outpatient setting is based on conjugation of a monoclonal antibody to human red blood cells with antibody to plant-like peptides or proteins based on an assay described by Kemp et al. (Kemp *et al.*, 1988). The red cells are

cross linked via the monoclonal antibody moiety, resulting in agglutination of the red blood cells in the blood sample if the antigen or antibody to the parasite component is present in the blood sample. ELISA and PCR can be utilized with samples collected on filter paper as is standard in Newborn Screening Programs and also facilitates outpatient and field use.

Example 34. <u>Development and use of Antisense Oligonucleotides in Design and Use of Medicines to Protect Against Apicomplexans:</u>

Antisense oligonucleotides directed against the nucleic acids which encode the enzymes of the essential parasite metabolic process described herein are effective medicines to treat these infections. Antisense oligonucleotides also are directed against transit sequences in the genes. Antisense oligonucleotides are short synthetic stretches of DNA and RNA designed to block the action of the specific genes described above, for example, chorismate synthase of *T. gondii* or *P. falciparum*, by binding to their RNA transcript. They turn off the genes by binding to stretches of their messenger RNA so that there is breakdown of the mRNA and no translation into protein. When possible, antisense do not contain cytosine nucleotides. Antisense reagents have been found to be active against neoplasms, inflammatory disease of the bowel (Crohn's Disease) and HIV in early trials Antisense will not contain cytosine nucleotides followed by guanines as this generates extreme immune responses (Roush, 1997). Antisense oligonucleotides with sequence for thymidine kinase also is used for regulatable gene therapy

Example 35. <u>Ribozymes and Other Toxic Compounds as Antimicrobial Agents:</u>

Ribozymes are RNA enzymes (Mack, McLeod, 1996) and they and toxic compounds such as ricins (Mahal et al. 1997) are conjugated to antisense oligonucleotides, or intracellular antibodies, and these constructs destroy the enzyme or other molecules.

Example 36. <u>Intracellular Antibodies to Target Essential Enzymes Proteins and Organelles:</u>

Intracellular antibodies are the Fab portions of monoclonal antibodies directed against the enzymes of this invention or portions of them (e.g., anti-transit sequence antibodies) which can be delivered either as proteins or as DNA constructs, as described under vaccines.

Example 37. <u>Development of New Antimicrobial Compounds Based on Lead Compounds:</u>

The herbicide inhibitors comprise lead compounds and are modified as is standard. Examples are where side chain modifications or substitutions of groups are made to make more active inhibitors (Table 1). Their mode of action and structure as well as the enzyme and substrate structures are useful in designing related compounds which better abrogate the function of the enzymes Examples of such substrate or active site targeting are listed in Table 1.

Native or recombinant protein used in enzymatic assays and *in vitro* assays described above are used to test activity of the designed newly synthesized compounds Subsequently, they are tested in animals

Example 38. Trials to Demonstrate Efficacy of Novel Antimicrobial Agents for Human Disease:

Trials to demonstrate efficacy for human disease are performed when *in vitro* and murine and primate studies indicate highly likely efficacy and safety. They are standard Phase I (Safety), Phase II (small efficacy) and Phase III (larger efficacy with outcomes data) trials. For medicines effective against *T. gondii* tachyzoites, resolution of intracerebral *Toxoplasma* brain lesions in individuals with HIV infection with no other therapeutic options available due to major intolerance to available medicines is the initial strategy for Phase II trials. Endpoints for trials of medications effective against *T. gondii* bradyzoites include absence of development of toxoplasmic encephalitis in individuals with HIV. HIV infected patients who also are seropositive for *T. gondii* infection are evaluated. Evaluation is following a one-month treatment with the novel anti *T. gondii* medicines. Observation is during a subsequent 2-year period when the patients peripheral blood CD4 counts are low. Effective medicines demonstrate efficacy measured as absence of *T. gondii* encephalitis in all patients. Otherwise, 50% of such individuals develop toxoplasmic encephalitis. When medications efficacious against bradyzoites and recrudescent toxoplasmic encephalitis in patients with AIDS are discovered and found to be safe, similar trials of efficacy and safety for individuals with recurrent toxoplasmic chorioretinitis are performed. All such trials are performed with informed consent, consistent with Institutional NIH, and Helsinki guidelines applicable to treatment trials involving humans

Example 39. Vaccine Trials for Humans

After vaccine efficacy in rodent models to prevent congenital and latent *Toxoplasma* infection are established, for component vaccines only, trials to establish safety and efficacy in prevention of congenital and latent infection are performed. They follow standard procedures for phase I, II and III trials as outlined above and as is standard for vaccine development.

Endpoints for vaccine effect and efficacy are development of antibody and cell-mediated immunity to *T. gondii* (effect) and most importantly, prevention of *T. gondii* congenital infections. After establishing in phase I trials that the vaccine is entirely safe, nonpregnant women of childbearing age will be vaccinated with recombinant vaccine. Assay for efficacy is via a serologic screening program to detect newborn congenital toxoplasmosis (described in Boyer and McLeod, 1996) with usual testing to document whether seropositive infants are infected (described in Boyer and McLeod, 1996).

Example 40. Vaccine Efficacy and Safety for Livestock Animals

The efficacy of candidate vaccines is tested in sheep as previously described (Buxton *et al.*, 1993). Vaccines are live attenuated, genetic constructs or recombinant protein. The most efficious routes and frequency of inoculation is assessed in a serious of experiments as described below. Intra-muscular, sub-cutaneous and oral are the preferred routes, although intravenous, intraperitoneal and intradermal routes may also be used. Scottish blackface or/and swaledale ewes, four to six years old are tested for IgG antibodies to *Toxoplasma gondii* using an ELISA assay. Only sero-negative

animals are used for the study. Three groups of 10-15 ewes are used for each experiment. Groups 1 are vaccinated, while group 2 and 3 are not. Three months later all ewes are synchronized for estrous and mated. At 90 days gestation the ewes in groups 1 and 2 are given 2000 sporulated oocyst of *T. gondii*.

The outcome of pregnancy is monitored in all groups. Aborted lambs or those dying soon after birth are examined histologically and by PCR for the B1 gene or sub-inoculation into mice or tissue culture, for the presence of *T. gondii*. All placentas are examined histologically and as above for parasites. Lambs are weighed at birth. Pre-colostral serum is taken from each lamb. Congenital transmission is assessed by performing ELISA assays on the serum for IgG or IgM. Protection is measured as a decrease in congenital transmission, a decrease in the incidence or severity of congenital disease, or a decrease in abortion.

Example 41: *T. gondii*Chorismate Synthase Genomic Sequence is Used to Produce "Knockouts" (Attenuated Vaccine Strain).

The genomic sequence of chorismate synthase is in FIG. 13. As with other genomic sequences herein. it provides an example of a gene which is "knocked out" to produce an attenuated vaccine and also can be utilized as described in other parts of this document.

A chorismate synthase knock out parasite was produced as follows: The genomic *T. gondii* chorismate synthase sequence consists of 9 exons. To prepare the knockout construct, this sequence was digested with EcoN1 to remove a 1.8 kb fragment that included exons 2, 3, and 4. The EcoN1 digested ends were blunt ended followed by dephosphorylation. A 1.9 kb piece bearing HXGPRT flanked by the 5' promoter region and 3' untranslated region of dhfr (called dhfr HXGPRT) was isolated by digestion of a construct, obtained from J. Boothroyd, and XbaI and hoI. After blunt ending, the 1.9 kb fragment was cloned into the chorismate synthase construct so that dhfr HXGPRT replaced chorismate synthase exons 2, 3 and 4. This construct was used for knockout of the wild type chorismate synthase gene.

The sequence of the construct was verified by PCR. Following transfection into *T. gondii* (deficient in HXGPRT) and selection in medium containing 25 μg/ml mycophenolic acid and 50 μg/ml Xanthine, successful transfection was confirmed by PCR of the chorismate synthase/dhfr HXGPRT junction and sequence the product. Parasites were cloned by limiting dilution and clones were cultured in the presence or absence of folate and other aromatic products in this medium with replica cultures. Aromatic compound deficient medium with 10% AlbuMax® as a serum substitute was prepared. Final concentrations of aromatic compounds in the supplemented medium are 0.1M phenylalanine, tyrosine, tryptophan, PABA, 2,3 dihydroxybenzoate and p-hydoxybenzoate. DNA was extracted from those replicate cultures of parasite clones that grew only in the presence of aromatic compound

supplementation. PCR primers were designed to confirm presence of the knockout construct and demonstrated that homologous recombination occurred resulting in replacement of exons 2-4 with the dhfr HXGPRT sequence. The knockout parasite was passaged in aromatic compound supplemented medium. Whether this selection clearly demonstrates inability of the knockout parasite to grow in aromatic compound deficient medium, but ability to grow in aromatic compound sufficient medium using a uracil assay. Such *aro* deficient strains of bacteria have been used as vaccines precisely because they are nonpersistent. Complementation with *aroC* in an episomal vector to prove that the phenotype of the chorismate synthase knockout organisms is due to deletion of the chorismate synthase gene, was also done. This complementation system also allows characterization of the effects of mutations in chorismate synthase or its promoter region on transcription or on enzyme function, importance of the pathway for parasite viability, stage switch and subcellular localization. An episomal vector was obtained from John Boothroyd. Chorismate synthase was cloned within this plasmid under control of a constitutive promoter (e.g., the promoter for tubulin or DHFR). The resulting construct was transfected into the chorismate synthase knockout parasite described above. Proof that the construct produces mRNA for chorismate synthase is with northern and western blotting. The lack of ability of the knockout and the ability of the complemented parasite to grow in folate and other aromatic compound deficient medium indicates a functional construct. This knockout organism is suitable for use as an attenuated vaccine strain.

Example 42: *T. gondii* Chorismate Synthase cDNA Sequence in a DNA Vaccine Vector Elicits Antibodies.

T. gondii chorismate synthase cDNA sequence placed in a DNA vaccine vector with a CMV promoter (Vical, San Diego) and administered intramuscularly to mice elicits serum antibodies to chorismate synthase (FIG. 14 A and B). Antibody production is detected on Western blot and in other immunoassay systems. This is an example of

a recombinant vaccine and a system to produce antibody reagents useful in diagnostic tests without the need to produce recombinant protein.

Example 43: <u>*T. gondii* Chorismate Synthase-green Fluorescent Protein Construct is Made and Used in Parasite Survival Assays to Test Antimicrobial Agents.</u>

A *T. gondii* chorismate synthase-green fluorescent protein DNA construct elicits a fusion (reporter) protein detectable with conventional immunofluorescence microscopy and deconvolution microscopy (FIG. 15) and other techniques known in the art to detect fluorescence. This construct accelerates the growth rate of the parasite and is useful for measuring effects of antimicrobial agents on the parasite by detecting relative amounts of the green fluorescent reporter protein. This is useful for testing antimicrobial agents.

Example 44: <u>Chorismate Synthase and Life Cycle.</u>

Chorismate synthase is differentially located and expressed in different life cycle stages indicating that it can be an antimicrobial agent target in, and reagent to detect, specific stages of the parasite.

<u>Immunostaining</u> This is performed as is standard in the art with tachyzoites, converting organisms, intestinal life cycle stages using specimens produced *in vivo* and *in vitro*.

In some tachyzoites, chorismate synthase was concentrated in a small area contiguous to the nucleus in the area of the plastid (FIG. 16A). In other life cycle stages it was distributed diffusely throughout the cytoplasm (FIG. 16B, C). It was most abundant in bradyzoites and macrogametes. A C-terminal green fluorescent protein reporter alters its localization in tachyzoites (FIG. 15). Unique stage-associated expression and subcellular localization of *T. gondii* chorismate synthase is identified in tachyzoites, bradyzoites and in the stages of the parasite in the cat intestine including macrogametes, microgametes but not schizonts.

Stage-associated expression of *T. gondii* chorismate synthase (FIG. 16A-C) is an example of the expression and differential subcellular localization of this protein. This stage-associated expression demonstrates

that this protein is present in tachyzoites (A), bradyzoites (B) and microgametes (C) and macrogametes (C). This is an antimicrobial agent target, useful diagnostic reagent and vaccine constituent for infections with these life cycle stages. The differential stage associated subcellular localization demonstrates that organelle targeting is another way to target these enzymes.

Example 45: Recombinant Chorismate Synthase is Useful for Antibody Production and in Enzyme Assays for High Throughput Screens.

Recombinant chorismate synthase was produced and is useful for high throughput screens, development of diagnostic reagents and a vaccine.

Overexpression of Chorismate Synthase Chorismate synthase was expressed in *E. coli* using a pGEX expression system (Pharmacia). Briefly, PCR was used to amplify the coding region and to introduce BamH1 and EcoR1 sites to the 5' and 3' ends respectively. Following removal of the 3'adenosine overhangs, the PCR product was first cloned into pUC18 using the Sureclone Ligation Kit (Pharmacia Biotech, Herts, UK). The pUC18 plasmid containing the insert was digested with EcoR1 and BamH1 and following purification by electrophoresis, the insert was eluted from an agarose gel and then cloned into pGEX-2T. DNA sequencing confirmed that the nucleotide sequence was in frame and that no PCR errors had been introduced. Following transformation the protein was expressed in BL21. To optimize expression and to test protein for enzymatic activity, expression is increased using BL21 Codon Plus (Stratagene). This strain of *E. coli* has been engineered to contain extra copies of tRNAs for codons in *E. coli* that are rarely used (*arg*U, *ile*Y, *leu*W and *pro*L). In some cases the presence of an N-terminal tag can interfere with the ability of a protein to function and that although a GST tag can be removed with thrombin this treatment itself can be too harsh to retain the activity of some proteins. Thus as an alternative approach is to employ the Protein C Epitope Tagging system (Roche). This system

allows the production of recombinant proteins which have either C-terminal or N-terminal protein C tags. The protein C tag is used to purify protein using an antibody that binds the protein C tag only in the presence of Ca^{+2}. Calcium chelation then provides a gentle means of eluting the purified protein from the antibody.

The *Pichia* Expression System (Invitrogen) is also used. This system offers advantages of bacterial systems such as high-level expression and ability to use large scale cultures. In addition, it offers certain advantages of eukaryotic expression systems that facilitate protein processing, folding and post-translational modifications. The system makes use of the powerful alcohol oxidase promoter (AOX1) to aid high expression levels. Tranformants are selected by Zeocin resistance and inframe C-terminal His tag allows purification by metal-chelating resins and detection through an anti-*myc* antibody. This produces additional recombinant chorismate synthase protein, in order to produce-polyclonal antisera to chorismate synthase. Antisera is employed to determine subcellular localization of *T. gondii* chorismate synthase. Recombinant protein also is used for later crystallography studies and for high throughput screens.

<u>Production of anti-chorismate synthase antibody</u> To produce polyclonal antiserum to the entire protein, mice with 10 ug of recombinant protein emulsified with TiterMax initially and then again 2 weeks later. A commercial source for immunization of rabbits is also suitable. Preimmune sera and sera containing polyclonal antibody, is obtained 7 days after the second immunization. To produce monospecific antibody, anti-peptide antibodies to specific regions of the protein also is produced in rabbits by a commercial laboratory (Alpha Diagnostic, San Antonio, TX). Analysis for B cell epitopes indicates that amino acids 342 to 363, KHERDGCSAATLSRER ASDGRT, and amino acids 35 to 55, SVEDVQPQLNRRRPGQGPLST are peptides that should elicit monospecific antibodies. The advantage of polyclonal antibodies is that

they recognize native folded protein. and of the anti-peptide antibodies is that when they recognize native protein. peptide epitopes are defined. Development of enzyme assay for high throughput assays To measure chorismate synthesis, a phosphate release assay is performed using a malachite green dye and the product is detected spectrophotometrically with a plate reader. This is adapted for large scale screening for high throughput screens. This assay is performed anaerobically (i.e., in a nitrogen environment) using polyethylene bags. Substrate EPSP will be synthesized as described previously.

Example 46: Antibody to Recombinant Chorismate Synthase is Useful in Diagnostic Assays.

Antibody to recombinant chorismate synthase was produced in mice and is useful as an immuno-diagnostic test kit reagent.

Example 47: Isocitrate Lyase.

T. gondii isocitrate lyase activity was demonstrated and has the same uses as chorismate synthase activity, and other enzymes, e.g., it is useful for high throughput screens of *T. gondii*, Isocitrate lyase enzyme activity (FIG. 17C, D) and its inhibition by 3 nitropropionic acid (3NPA) (FIG. 17D) was identified. This exemplifies the presence of a key enzyme in the glyoxylate cycle, and provides a method useful for both screens of available libraries of compounds and rational development of combinatorial libraries of compounds based on lead compounds and their interactions with the enzme and analysis of enzyme structure. Use of a knockout microorganism complemented with the parasite ICL gene is another example of a method useful for high throughput screens to idnetify an inhibitor of ICL. sequences Antisense gene sequences to interfere with parasite growth or survival. This is a representative example of inhibition of this enzyme in this pathway. This enzyme is potentially useful in development of antimicrobial agents, diagnostic reagents or vaccines.

Example 48: <u>The *T. gondii* Isocitrate Lyase Binding Pocket and Active Site Form a Basis for Rational Antimicrobial Agent Development.</u>

The *T. gondii* isocitrate lyase cDNA sequence (FIG. 18), amino acid sequence (FIG. 19), and isocitrate lyase binding pocket and active site (FIG. 20, box) were identified and have absolute homologies with all other isocitrate lyases and not with other partially homologous enzymes such as CPEP mutase. A yeast with a mutation in a base encoding a lysine (K) only in this area produced an inactive isocitrate lyase. This observation is useful for development of antimicrobial agents as described for other sequences herein.

Example 49: <u>*T. gondii* Isocitrate Lyase Genomic Sequence is Useful for Vaccine Development</u>

A genomic ICL sequence is in FIG. 21 and is useful for vaccine development as described for thoer genomic sequences.

Example 50: <u>Demonstration of *T. gondii* Isocitrate Lyase Stage Associated Protein and mRNA.</u>

T. gondii isocitrate lyase stage associated protein is present in bradyzoites and is useful as described herein for producing diagnostic reagents, identifying anti-microbial agents and for vaccines. T. *gondii*, isocitrate lyase stage-associated protein is present in bradyzoites (FIG. 22) and there is stage associated mRNA expression and protein (FIG. 23). This observation is useful in the same manner as other examples of mRNA and protein described herein in for diagnostic reagents, antimicrobial agent and vaccines.

Example 51: <u>Additional Inhibitors of Apicomplexan Isocitrate Lyase are Based on Compounds that Inhibit Isocitrate Lyases of Other Organisms.</u>

Additional inhibitors of apicomplexan isocitrate lyases are identified and designed. They are used as lead compounds for designing new inhibitors as described herein and this is useful for development of

diagnostic reagents, antimicrobial agents and vaccines as described for other enzymes herein.

Example 52: <u>Genetic, Enzymatic and Functional Evidence and Active Inhibitors of Apicomplexan Acetyl coA Carboxylases Such as Clodinafop Provide a Basis for Development of Novel Antimicrobial Agents, Diagnostic Reagents and Vaccines.</u>

FIG. 24 presents enzymatic, genetic and functional evidence of a wheat-like *T. gondii* acetyl coA carboxylases. Partial gene sequences were identified for *T. gondii*, Plasmodia and Cryptosporidia acetyl coA carboxylases. Inhibitors of *T. gondii* acetyl coA carboxylase inhibited parasite survival in vitro. This is useful for diagnostic reagents, antimicrobial agents and vaccines as described for other sequences herein.

Example 53: <u>Synergism of Antimicrobial Agents that Inhibit Apicomplexan Lipid Synthesis.</u>

Other examples of synergistic effects on lipid synthesis pathway are the synergistic effects of clodinofop, thialactomycin, and cerulin.

Example 54: <u>Growth of *Toxoplasma gondii* is Inhibited by Aryloxyphenoxy-propionate Herbicides Targeting Acetyl-CoA Carboxylase.</u>

The recently discovered plastid-like organelles in apicomplexan parasites provide new targets for antimicrobial agents. Aryloxyphenoxypropionates, known inhibitors of the plastid Acetyl-CoA Carboxylase (ACC) of grasses, inhibit *Toxoplasma gondii* ACC by 50% at a concentration of 20 μM Clodinafop, the most effective of the herbicides

tested, inhibits growth of *T. gondii* in human fibroblasts by 70% at 10 μM and is not toxic to the host cell even at much higher concentrations. Infected fibroblasts treated with Clodinafop for two days show a substantial reduction in the number of *T. gondii* cells at 10 μM and almost complete removal of parasites at 100 μM. Longer treatments are even more effective. Fragments of genes encoding biotin carboxylase domain of multi-domain ACCs were cloned. One ACC from *T. gondii* (ACC1) clusters with the putative *Cyclotella cryptica* chloroplast ACC and *Plasmodium* ACC, while another (ACC2) clusters with *Cryptosporidium* ACC, probably the cytoplasmic form.

In plants, genes encoding enzymes for fatty acid synthesis, including various subunits of ACC except one, are present in the nuclear genome and their protein products are imported and function in plastids. ACC, catalyzing the first committed step of *de novo* fatty acid biosynthesis, is a known selective target of aryloxyphenoxypropionate ("fops") and cyclohexanedione ("dims") herbicides in sensitive species. The molecular mechanism of inhibition/resistance of the enzyme is not know but there is a strong correlation between the enzyme structure and its origin. The high molecular weight multi-domain ACC that is localized in plastids of grasses is extremely sensitive to these herbicides. All of the multi-subunit chloroplast enzymes of dicot plants and bacteria as well as other multi-domain cytosolic ACCs, such as those from man, chicken, rat and yeast, are resistant. ACC activity is conveniently measured *in vitro* by the incorporation of the carboxyl group from bicarbonate into an acid-stable form using crude protein extracts after Sephadex G50 filtration. Substantial, acetyl-CoA dependent activity was observed in extracts from tachyzoites of the RH strain of *T. gondii* isolated from infected mice, and no ACC activity could be detected in a control extract of macrophages from uninfected mice, the usual minor contaminant of the parasite preparation. Two biotin-containing proteins were revealed with streptavidin following electrophoresis of the extract proteins. One band at

240 kDa corresponded to the expected size for a subunit of ACC, while another at 130 kDA corresponded to the size expected for pyruvate carboxylase (PC).

Structures of fops and dims were tested on the ACC-containing protein extracts of *T. gondii* described above. Three of the four fops were striking inhibitors of the activity, while none of the dims had any effect against the enzyme. There was 50% inhibition at 20 μM and 90% inhibition at 100 μM by Clodinafop, Quizalofop, and Haloxyfop. Effects of the herbicides on uninfected fibroblasts and on *T. gondii* growth and replication were tested as previously described by Roberts *et al.*, 1998 using incorporation of radiolabeled thymidine by growing fibroblasts to assess toxicity and incorporation of radiolabeled uracil to measure *T. gondii* growth and persistence. Anti-parasite activity and toxicity for four fops and one representative dim were determined. Pyrimethamine and sulfadiazine, antimicrobial agents which are known inhibitors of folate synthesis, were included as positive control. The combination of candidates inhibited uracil incorporation by *T. gondii* by more than 95% without toxicity for fibroblasts. Consistent with the data for ACC activity *in vitro*, the inhibitory activity of the fops and the dim on *T. gondii* growth in fibroblasts was in the same concentration range. Clodinafop was even more active in this assay than in the enzyme assay, giving 70% inhibition at 10 μM. With regard to toxicity, fops are mildly toxic at the highest concentration, 400 μM. In separate experiments, the effect of Clodinafop on *T. gondii* was assessed by light microscopy. Micrographs showed infected fibroblasts treated with Clodinafop at 10 and 100 μM compared with control infected cells without herbicide and uninfected cells. There is substantial reduction of the number of *Toxoplasma* tachyzoites at 10 μM and almost complete removal at 100 μM. The effectiveness of Clodinafop at 10 μM is greatly enhanced by a 4-day treatment with one change of medium and inhibitor after 2 days. In this

experiment, cultures were incubated for 2 more days without the inhibitor. No parasite cells were found in infected fibroblasts treated in this way.

The active form of fops used as herbicides in the field are esters, which are converted to free acids by plant esterases. The true inhibitor of ACC is the free acid. Two esters of Halosyfop, two esters of Quialofop and one ester of Clodinafop (Topik) have no effort on *T. gondii* ACC activity in crude extracts and were relatively inactive in the uracil incorporation assay except for Topik that was as active as the free acid, suggesting significant level of hydrolysis of this ester. In general, in this assay fop esters are not more effective than free acids.

Single stranded cDNA prepared from total RNA extracted from *T. gondii* tachyzoites was used as a template for the PCR amplification of a 440-bp fragment encoding the biotin carboxylase (BC) domain of ACC, using primers and conditions described for wheat ACC. Several independent PCRs yielded five different products. Two of them appeared to encode eukaryotic-type multi-subunit ACCs. Genomic clones encoding the entire BC domain were then isolated from a genomic library using the PCR-cloned fragments as probes and these were sequenced. Similarly, sequences of a fragment of the BC domain of ACCs of *P. knowlesii*, *P. falciparum* and *C. parvum* were determined from PCR-cloned gene fragments. A phylogenetic analysis was performed based on amino acid sequence comparisons of the two candidate ACCs from *T. gondii* with those of other BC domains. Three apicomplexan sequences (*T. gondii, P. knowlesii,* and *P. falciparum*) cluster together with *Cyclotella cryptica* ACC, an enzyme thought to be in the diatom chloroplast. This isozyme, called ACC1 in *T. gondii*, is likely the plastid form. This assignment awaits cloning and sequencing of the 5'-terminal portion of the cDNA, where a sequence encoding a signal/transit peptide ought to be found. The other ACC, called ACC2 in *T. gondii*, clusters with the ACC of *C. parvum*. These two are probably cytosolic forms. The partial genomic

sequences revealed differences in intron number and location before *ACC1* and *ACC2* of *T. gondii*, and the three ACC genes from the other apicomplexa.

One of the other PCR products encoded a BC domain similar to that of pyruvate carboxylases. Deduced amino acid sequences encoded by the remaining two PCR products were similar to the BC domains of rat ACC and prokaryotic-type biotin-dependent carboyxlases, respectively. These fragments were assumed to encode the host mouse ACC and a carboxylase from a bacterial comaminat. Protein gels blotted with streptavidin revealed pyruvate caroxylases (130 kDa) in addition to ACC (240 kDa), but no bacterial-type biotin carboxyl carrier protein (20 kDa) or biotinylated subunit of propionyl-CoA carboxylase (70 kDa).

There is a very strong correlation between the pattern of sensitivity/resistance of the ACC activity and *Toxoplasma* growth inhibition by the twelve different compounds tested. This result provides important evidence linking the *Toxoplasma* growth phenotype to the effect of the compounds on the enzyme activity. The basis for the sensitivity of some of the multi-domain ACCs to fops and dims is not known, nor is it known why some, like the *T. gondii* ACC activity reported here, are sensitive to fops but resistant to dims. Compounds in the fop family differ in their properties as well, with a clear correlation between activity and structure, e.g. relatively low inhibitory activity of Fluazifop.

The target for sensitivity (herbicide binding site) is likely in to a region encompassing the β domain of carboxytransferase, based on experiments using yeast gene replacement strains, in which chimeric genes encoding wheat ACCs replace the yeast *ACC1* gene. Such strains are herbicide-sensitive if they contain a gene encoding sensitive ACC. Availability of the genes encoding *T. gondii* ACCs may clarify which of the isozymes is targeted to the plastid and whether one or both of them are sensitive to fops (the majority of the activity in the protein extracts was inhibited).

Inhibition of *T. gondii* growth in infected fibroblasts by herbicides targeting ACC suggests, based on earlier studies of herbicide action on plants and yeast gene-replacement strains, that inhibition of ACC activity in sensitive species leads to metabolite depletion to a level at which the organism cannot support its needs. This reflects an essential contribution of ACC to the pathway of *de novo* fatty acid synthesis and is the basis for the use of the ACC inhibitors as herbicides in agriculture and their potential future use in medicines.

Example 55: <u>An Apicomplexan Glyoxylate Cycle</u>.

To determine whether there are additional plant-like metabolic pathways as potential targets for novel chemotherapeutic agents, because they are not present in animals or differ substantially from those of animals, evidence was sought that the glyoxylate cycle might be operational in apicomplexan parasites, and play an essential role in certain stages of the life-cycle of these organisms.

Evidence was sought for the presence of isocitrate lyase and malate synthase, key enzymes unique to the glyoxylate cycle. Enzymes of the glyoxylate cycle were detected in protein extracts of *T. gondii*. Polyclonal antibodies to cotton malate syntase and isocitrase lyase were used to detect heterologous apicomplexan proteins by western blot analysis. A protein band of approximately 64 kD was detected using antibodies to cotton isocitrate lyase and malate synthase in lysates of *T. gondii* tachyzoites. Isocitrate lyase was also sought, and found in western blots of *T. gondii* bradyzoites. Antibody to cotton isocitrate lyase also was used for immunohistochemistry to study bradyzoites within cysts in brain tissue. Isocitrate lyase was identified in bradyzoites. Whether there was stage related expression of isocitrate lyase in *T. gondii* was studied by using smaller number of parasites in semiquantitative western blots. There was greater expression of isocitrate lyase in parasites undergoing stage conversion *in vitro* on the first and second

days of culture following pH shock, with loss of detectable isocitrate lyase protein on the third and seventh day with concomitant appearance of increasing levels of the bradyzoite marker BAG 1 as the bradyzoites matured when relatively small numbers of parasites were used. Stage specific expression of the gene was analyzed by RT PCR using mRNA obtained from Me49 strain *T. gondii* tachyzoites differentiating in bradyzoites *in vitro*. Tachyzoites had demonstrable ICL mRNA whereas bradyzoites did not. These results suggest that expression of isocitrate lyase may be developmentally regulated. In other microorganisms, isocitrate lyase is regulated at a number of different steps. For example, in *E. coli* there is an *ace* operon comprised of *ace B, A,* and *K* encoding malate synthase, isocitrate lyase and isocitrate dehydrogenase kinase phosphatase, respectively. Expression of the *ace* operon is under the transcriptional control of two genes, the *iclR* gene and *fadR*. The *fadR* is also involved in the regulation of fatty acid degradation. It has been suggested that these genes encode repressor proteins, which act independently or in concert, to repress the *ace* operon. Moreover, functionally related isoenzymes with distinct roles in the metabolic pathways needed for growth under different minimal conditions also have been described. In addition, different isoforms of the isocitrate lyase enzyme related to the age of the organism have been identified. Interestingly, in germinating seeds, isocitrate lyase plays a time-limited role with decline in isocitrate lyase activity in the senescent endosperms.

Next, evidence for the presence of a functional glyoxylate cycle enzyme and its inhibition in apicomplexan parasites was obtained isocitrate lyase enzyme activity and its inhibition by 3 Nitropropionic acid (NPA) was detected in lysates of *T. gondii* tachyzoites. Functional evidence for the glyoxylate cycle was sought by examining the effects of inhibitors of isocitrate lyase on growth and survival of apcomplexan parasites *in vitro*. Uracil incorporation by *T. gondii* in the presence and absence of inhibitor was

measured in tachyzoites. 3 NPA inhibited parasite growth. Similarly, 3NPA inhibited growth of *P. falciparum*.

Then, genetic evidence for the presence of isocitrate lyase was obtained in *T. gondii*. First the primary structure of isocitrate lyases from varied organisms (bacteria to higher plants) were compared, and absolutely conserved amino acid sequences were identified across species. A partial complementary DNA sequence was next identified from the WashU-Stanford-PAMF-NIH *Toxoplasma* EST project (EST TgESTzz53c08.rl; GenBank accession number AA520237; Steve Parmly, PAMF, www.ncbi.nln.nih.gov/Malaria/plasmodiumbl.html). Both strands of the corresponding clone were sequenced. This sequence when translated had an open reading frame (ORF) of 857 base pairs, had over 30% homology with isocitrate lyases from varied organisms (range: 29 - 53% identities; 43 - 67% positives). A *T. gondii* RH strain genomic Lambda DASH II library (Stratagene) was then screened using TgESTzz53c08.r1 as a probe, and a genomic clone was obtained and sequenced (GenBank accession number to be assigned). The binding pocket and catalytic site that are absolutely conserved among other isocitrate lyases was identified in the *T. gondii* gene. The deduced amino acid sequence also showed partial homology with putative carboxyphosphonoenolpyruvate phosphonomutase from *E. coli* and *Salmonella* species. Two regions of isocitrate lyase have been implicated as part of the active site. The motif KKCGHM(L) is conserved in all isocitrate lyases, and it is proposed that the cysteine is a critical active site residue. The absolute identity of the *T. gondii* sequence in the region of the active site, the binding pocket and other conserved regions to that of all isocitrate lyases, not demonstrated by any carboxyphosphonoenolpyruvate phosphonomutase, makes it highly likely that the gene cloned is an isocitrate lyase gene. Also, interestingly, a single

mutation of a K to R at the second lysine in the KKCGHM(L) motif (a substitution noted in a number of carboxyphosphonoenolpyruvate phosphonomutase genes) in a yeast and *E. coli* isocitrate lyase rendered it inactive (Figure 4B)[14-16]. The putative *T. gondii* isocitrate lyase gene sequenced thus far has predicted 4 exons.

These studies provide protein, enzymatic, functional and genetic evidence for the presence of a glyoxylate cycle in apicomplexan parasites. The presence of the glyoxylate cycle pathway enzymes, but not expression of its mRNA appears to be more abundant in certain life cycle stages of *T. gondii* in which lipids may be utilized in preference to carbohydrates as an energy source. This pathway provides a novel antimicrobial agent target and an inhibitor of an enzyme in this pathway has been identified.

MATERIALS AND METHODS

T. gondii

Swiss Webster mice (12-15 mice per assay) were infected intraperitoneally with *T. gondii* tachyzoites (Rh strain, $2x10^7$ per mouse) 2 days prior to assay. Tachyzoites were extracted with a peritoneal lavage using 5 ml of sterile saline per mouse.

Alternatively, the PTg strain of *T. gondii* was cultured as tachyzoites or tachyzoites induced to become bradyzoites, as described[8].

Antibodies

Rabbit control preimmune serum was obtained and then antibodies to cotton malate synthase or isocitrate lyase were produced in rabbits

SDS PAGE and Western blots

T. gondii tachyzoites or bradyzoites were obtained at indicated time points from host cells by scraping the monolayer, passing the infected cells through a syringe with a 25g needle twice to disrupt them, and then organisms were counted and centrifuged at 2000 rpm for 10 minutes at 4°C to pellet the parasites. The supernatant was discarded and the pellet was suspended in SDS PAGE loading buffer (with 2 mercaptoethanol) at a

concentration of 1×10^5 parasites per µl and boiled for 10 minutes. Unless otherwise indicated, material from 2×10^6 parasites was utilized per lane. This was electrophoresed in a 12% polyacrylamide gel under reducing conditions and transferred onto nitrocellulose membranes blocked with 5% milk in PBS tween (0.05%), and probed with rabbit preimmune serum or polyclonal antibody to cotton isocitrate lyase or malate synthase, or mouse monoclonal antibody to BAG1 antigen, followed by HRP conjugated anti rabbit or anti mouse secondary antibodies as appropriate. Bands were visualized using ECL.

PCR and Northern blots

Messenger RNA, isolated on oligo dT solid phase matrix columns and reverse transcribed using a random priming method, was used for semi-quantitative PCR analysis of tachyzoite surface antigen (SAG)1, bradyzoite cytosolic antigen (BAG)1-5, and isocitrate lyase (ICL), relative to beta tubulin (TUB). The primer sets were as follows: SAG1 (5'-CGG TTG TAT GTC GGT TTC GCT-3' and 5'-TGT TGG GTG AGT ACG CAA GAG TGG-3'), BAG1-5 (5'-CCC ATC GAC GAT ATG TTC GAG-3' and 5'-CGT AGA ACG CCG TTG TCC ATT G-3'), ICL (5'-TTG CCG TTC TGG AAA GCT AGT AAG A-3' and 5'-GCA AAC GCT GGT CCT CAA TGT-3'), and TUB (5'-GTT TCC AGA TCA CCC ACA GTC TTG G-3' and 5'-GAG CAA ACC CAA TGA GGA AGA AGT G-3'), yielding PCR product sizes of, 346, 225, 574, and 420bp, respectively. The BAG1-5 primers flank an intron serving as a control for genomic DNA contamination, yielding a PCR product of 784 bp. cDNA from *T. gondii* tachyzoites of the RH strain and induced bradyzoites from the Me49 strain were used as templates.

Immunohistochemistry

Immunoperoxidase staining was performed as previously described using control preimmune or immune rabbit antisera.

Enzyme assays

Parasite lysates were obtained from tachyzoites, suspended in elution buffer (100 mM KCl. 20% glycerol, 7 mM 2-mercaptoethanol, 20 mM Tris-HCl, pH 7.5. and

complete TM protease inhibitor cocktail [Boehringer Mannheim, 1 tablet per 50 ml buffer], sonicated 3 times for 3 seconds at 30 sec intervals, and centrifuged at 12,000 g for 15 min. The supernatant collected was applied to a Sephadex® G100 column (25 ml, Pharmacia) equilibrated with elution buffer, eluted with 15 ml of elution buffer, and ≈1.5 ml fractions were collected. Fraction(s) with the peak protein concentrations (protein analysis performed on a spectrophotometer at 280 nm) were selected and used in enzyme assays.

A discontinuous method described by Ko and McFadden[17] was employed with minor modifications to measure the ability of isocitrate lyase to convert isocitrate to succinate and glyoxylate. This method utilizes the colorimetric reaction between the phenylhydrozone of glyoxylate and ferricyanide. Reaction mixtures (92 mM MOPS, 5 mM $MgCl_2$, 1 mM DTT, 1% phenylhydrazine, 4.4 mM isocitrate, in 0.5 ml with fractionated parasite lysate) were incubated in a 37 °C water bath for a determined amount of time. After incubation, enzymatic reactions were stopped with concentrated HCl, mixed with 25% (w/v) potassium ferricyanide, and then measured in a spectrophotometer at 520 nm.

Culture of parasites *in vitro* with inhibitors

Parasites were cultured with host cells and inhibitors and the effects analyzed as described[1].

Identification of *T. gondii* isocitrate lyase genes:

Library screening, phage DNA purification, Southern blot (cloning and sequencing), host strains and vectors

XL 1 Blue MRA and pBluescript KS⁺ DH5 α were used. Lambda Dash II (Stratagene) was the vector for the genomic library. A 550 bp ECOR1-XhoI fragment of the cDNA EST clone TgZZ13 CO8 r 1 was labeled with α (^{32}P) dCTP and used for initial screening of the library. For subsequent secondary and tertiary screening to obtain pure phage, a biotinylated, non-radioactive, labeled probe of the entire 857 bp EST clone was prepared and used. The genomic library was screened (Stratagene), phage purified to >99% homogeneity, the clone amplified and DNA extracted (Current Protocols in Molecular Biology).

Southern blot

The purified phage DNA was digested with NotI; xhoI or EcoRI enzymes, run on a 1x agarose gel, transferred onto a nylon membrane probed with the biotinylated probe (above). A ~4kb band which was identified with the probe and was cloned into pBluescript KS^{+} and sequenced.

DNA sequencing and sequence analysis

DNA sequencing was performed using an automated DNA sequencer. This sequence was compared to peptide sequence databases at The National Center for Biotechnology Information (NCBI) using the program TBlastX or BlastP (for derived open reading frames). Gene construction using the sequence obtained was also performed utilizing the Baylor College of Medicine program. Primers for sequencing were made at Integrated DNA Technology. Sequence analysis was carried out by software programs MacVector, ClustalX and MACH Box.

MATERIALS AND METHODS

A. Methods to Assay Candidate Inhibitors

1. *Inhibitors of Toxoplasma gondii*

a) Cell lines: Fibroblasts. Human foreskin fibroblasts (HFF) are grown in tissue culture flasks in Iscoves' Modified Dulbecoes Medium (IMDM), containing 10% fetal bovine serum, L-glutamine and penicillin/streptomycin at 37°C in 100% humidity and a 5% CO_2 environment. Confluent cells are removed by trypsinization and washed in IMDM. They are used in a growth phase for toxicity assays or when 100% confluent for parasite inhibition assays.

b) Tachyzoites: Tachyzoites of the RH and pTg strains of *T. gondii* are passaged and used for *in vitro* studies (McLeod *et al.*, 1992). The R5 mixed tachyzoite/bradyzoite mutant was derived from mutagenesis with nitrosoguanidine in the present of 5-hydroxynapthoquinone. These organisms are used for *in vitro* experiments at a concentration of 2 x 10^3, 2 x 10^4, or 2 x 10^5 organisms per ml, dependent upon the planned duration of the experiment (*i.e.*, larger inoculations for shorter duration experiments).

c) Bradyzoites: Bradyzoites are obtained as described by Denton *et al.* (1996b). Specifically, C57BL10/ScSn mice are infected intraperitoneally with 20 cysts of the Me49 strain of *T. gondii*. Their brains are removed 30 days later and homogenized in PBS by repeated passage through a 21 gauge needle. Aliquots containing the equivalents of 3-4 brains are diluted in PBS and 6.5 mls of 90% percoll added to the mixture which is allowed to settle for 30 mins. 2mls of 90% Percoll is

then added as a bottom layer and the mixture centrifuged for 30 mins at 2500xg. The cysts are recovered from the bottom layer and a small portion of the layer above. After the removal of Percoll by centrifugation, the contaminating red blood cells are removed by lysis with water followed by the addition of 1 ml of 10xPBS per 9 ml brain suspension in water. Bradyzoites are released from the purified cysts by digestion in a 1% pepsin solution for 5 minutes at 37°C. This method routinely permits recovery of greater than 90% of the cysts present which yields approximately 100 bradyzoites per cyst. Bradyzoites are used at concentrations of $2x10^3$, $2x10^4$ and $2x10^5$ per ml in parasite growth inhibition assays. pH shock is also used to retain organisms in bradyzoite stage when such pH does not interfere with inhibitor activity.

d) Inhibitors: Inhibitor compounds are tested over a range of concentrations for toxicity against mammalian cells by assessing their ability to prevent cell growth as measured by tritiated thymidine uptake and inspection of the monolayer using microscopic evaluation. A range of concentrations that are non-toxic in this assay are tested for their ability to prevent the growth of *T. gondii* and also other Apicomplexans within these cells.

i.) Heme Synthesis: The inhibitor of the heme synthesis pathway, gabaculine (Grimm, 1990, Elliot *et al.*, 1990; Howe *et al.*, 1995; Mets and Thiel, 1989; Sangwan and O'Brian 1993; Matters and Beale, 1995) is used at a concentration of 20 mM [which has been demonstrated to be effective against tachyzoites of the RH and R5 strains] Other inhibitors of this pathway include 4 amino-5-hexynoic

acid and 4-aminofluoropentanoic acid which provide additional corroborative evidence that this pathway is present.

ii) Glyoxylate Cycle: The inhibitor of isocitrate lyase is 3 nitropropionic acid (ranging from 0.005 to 5 mg/ml *in vitro*).

iii) Alternative Oxidase *T. gondii* bradyzoites use unique alternative oxidases. Alternative oxidase is necessary and sufficient for bradyzoite survival. Methods to characterize plant alternative oxidases are described (Hill, 1976; Kumar and Söll, 1992; Lambers, 1994; Li *et al.*, 1996, McIntosh, 1994).

For the *in vitro* studies, cell lines that lack functional mitochondria are used. These cell lines are used to allow the study of inhibitors effective against the conventional or alternative respiratory pathways within the parasite, but independent of their effects on the host cell mitochondria. Two cell lines, a human fibroblast cell line (143B/206) lacking mitochondrial DNA, and the parental strain (143B) which poses functional mitochondria are used. These cell lines have been demonstrated to support the growth of *T. gondii* (Tomavo S and Boothroyd JC, 1995). SHAM, an inhibitor of the alternative respiratory pathway is used at concentrations between 0.25 and 2 μg/ml *in vitro*.

iv) Shikimate Pathway. For EPSP synthase, the inhibitor is N-(phosphonomethyl) glycine (concentrations of 3.125mM in folate deficient media).

e) Culture assay systems for assessing inhibitor effect:

i) Toxicity assays: Aliquots of cells (HFF) are grown in 96-well tissue culture plates until 10% confluent. Cells are incubated with various concentrations of drug for 1, 2, 4 and 8 days. Cultures are pulsed with tritiated thymidine (2.5 μCi/well) for the last 18 hours of the culture after which the cells are harvested using an automated cell harvester and thymidine uptake measured by liquid scintillation.

ii) *In vitro* parasite growth inhibition assays: Confluent monolayers of HFF cells, grown in 96-well plates are infected with *T. gondii* tachyzoites of the RH strain and serial dilutions of anti-microbial compound are applied 1 hour later. *T. gondii* growth is assessed in these cultures by their ability to incorporate tritiated uracil (2.5 μCi/well) added during the last 18 hours of culture. After harvesting cells with an automatic cell harvester, uracil incorporation is measured by liquid scintillation. Alternatively, confluent HFF cells are grown in the chambers of Labtech slides and parasite growth is assessed microscopically following fixation in aminoacridine and staining in 10% Giemsa (McLeod *et al.*, 1992).

f) Product rescue assays to evaluate specificity of the inhibitor: To attempt to demonstrate specificity of the site of action of the inhibitor, growth inhibition assays are performed in the presence of varying concentrations of product, e.g., in the case where gabaculine is the inhibitor, ALA is added simultaneously to determine whether product rescue occurs. This type of study is only interpretable when rescue is demonstrated because it is possible that exogenous "product" is not

transported into the *T. gondii* within host cells. For EPSP synthase, product rescue assay is performed with PABA.

g) Assays for synergy *in vitro*. This is an assay in which ≤ 50% inhibitory concentrations of two antimicrobial agents are added alone and together to determine whether there is an additive, synergistic or inhibitory interaction. All other aspects of this assay are as described herein.

2. ***Inhibitors of Cryptosporidia parvum***

C. parvum oocysts at 50,000/well were incubated with each drug (PRM=paromomycin which is the positive control, NPMG, gabaculine, SHAM, 8-hydroxyquinoline) at 37°C (8% carbon dioxide) on confluent MDBKF5D2 cell monolayers in 96 well microtiter plates. The level of infection of each well was determined and analyzed by an immunofluorescence assay at 48 hours using as an antibody *C. parvum* sporozoite rabbit anti-serum (0.1%), and using fluorescein-conjugated goat anti-rabbit antibody (1%). Data are expressed as mean parasite count/field when 16 fields counted at 10x magnification "s.d. of the mean. (FIG. 6)

3. ***Inhibitors of Plasmodium falciparum***

This assay is performed in folate deficient RPMI 1640 over a 66 hour incubation in plasma as described by Milhous *et al.* (1985). Both the W2 clone DHFR resistant phenotype and the D6 clone are used (Odula *et al.*, 1988) (Table 3).

4. ***Inhibitors of Eimeria tenella***

Susceptibility of *Eimeria tenella in vitro* is analyzed by a method similar to that described by McLeod *et al.*, 1992 or for *Cryptosporidium* as disclosed herein.

5. *In vivo studies, measurement of parasitemia of Toxoplasma gondii*

A method to measure the amount of parasitemia in mouse peripheral blood has been developed. Briefly, the target for PCR amplification is the 35 fold repetitive B1 gene of *T. gondii* and the amplification was performed using primers previously reported. In order to semiquantitate the PCR product and to avoid false negative results, a competitive internal standard is generated using a linker primer and the original B1 primers. Competitive PCR was performed by spiking individual reactions (containing equal amounts of genomic DNA) with a dilution of the internal standard. Since this internal control contains the same primer template sequences, it competes with the B1 gene of *T. gondii* for primer binding and amplification. The sensitivity of the PCR reaction in each sample can be monitored. Following competitive PCR, the PCR products are distinguished by size and the amount of products generated by the target and internal standard can be compared on a gel. The amount of competitor DNA yielding equal amounts of products gives the initial amount of target gene.

6. *Interpretation of Data/Statistical Analysis of Data:*

In vitro studies are performed with triplicate samples for each treatment group and a mean ± sd determined as shown in the FIGs. All *in vivo* studies utilize at least 6 mice per group Statistical analysis performed by Students' t-test or the Mann-Whitney U-test. A p value of ≤ 0.05, is considered statistically significant.

B. Western Blots Demonstrate Plant-Like Enzymes

Western analysis for GSAT, isocitrate lyase, malate synthase, alternative oxidase and EPSP is used to demonstrate the presence of plant-like enzymes in many

Apicomplexan parasites, *e.g.*, *Plasmodia*, *Toxoplasma*, *Cryptosporidia*, *Malaria* and *Eimeria*.

Tachyzoites and bradyzoites (McLeod *et al.* 1984, 1988; Denton *et al.*, 1996a, b), or their mitochondria and plastids are isolated as previously described. Equivalent numbers of tachyzoites and bradyzoites are separately solubilized in 2x sample buffer and boiled for 5 minutes. Samples are electrophoresed through a 10 percent SDS-polyacrylimide gel. Proteins are transferred to a nitrocellulose membrane at 4°C, 32V with 25mM Tris and 192mM glycine, 20% v/v methanol, pH 8.3. Blots are blocked in PBS (pH 7.2) containing 5% powered milk and 0.1% Tween 20 for 2 hours at 20°C. After washing in PBS (pH 7.2), 0.1% Tween 20, blots are stained with polyclonal or monoclonal antibodies specific for alternative oxidases in PBS (pH 7.2) containing 0.1% Tween 20 for 1 hour at 20°C. Following washing in PBS (pH 7.2) containing 0.1% Tween 20, blots are incubated with an appropriate secondary antibody conjugated to HRP at a dilution to be determined by methods known in the art. After further washes, binding is visualized by chemoilluminescence (Amersham).

Antibodies to various enzymes, *e.g.*, soybean GSAT, barley GSAT, synechococcus GSAT, plant and/or trypanosome alternative oxidase, cotton isocitrate lyase, cotton malate synthase, soybean malate synthase, petunia EPSP synthase were used to determine whether homologous enzymes are present in *T. gondii* tachyzoites, bradyzoites, mitochondrial and plastid enriched preparations. Antibodies used include monoclonal antibodies to *Trypanosoma bruceii* and Voo Doo Lily (Chaudhuri *et al.* 1996) alternative oxidase and polyclonal antibody to *Trypanosoma bruceii* alternative

oxidase. The hybridizations with antibodies to plant and related protozoan alternative oxidases demonstrated the relatedness of *T. gondii* metabolic pathways to those of plants and other non-Apicomplexan protozoans. The products GSAT and alternative oxidase were demonstrated by Western analysis. Both polyclonal and monoclonal antibodies were reacted with alternative oxidase to confirm this observation.

C. <u>Probing Other Parasite Genes.</u> The genes isolated from *T. gondii* as described herein are used to probe genomic DNA of other Apicomplexan parasites including *Plasmodia*, *Cryptosporodium*, and *Eimeria*.

D. <u>Genomic Sequence</u>. Genomic clones are identified and sequenced in the same manner as described above for cDNA except a genomic library is used. Analysis of unique promoter regions also provide novel targets.

E. <u>Enzymatic Activity Demonstrates Presence of Plant-Like Enzymes in Metabolic pathways</u>

The presence of the enzymes putatively identified by inhibitor studies is confirmed by standard biochemical assays. Enzyme activities of GSAT, isocitrate lyase. malate synthase, alternative oxidase, and EPSP synthase, chorismate synthase, chorismate lyase, UDP-glucose starch glycosyl transferase and other enzymes listed herein are identified using published methods. Representative methods are those of Jahn *et al.*, 1991; Weinstein and Beale, 1995; Kahn *et al.*, 1977; Bass *et al.*, 1990; Mousdale and Coggins (1985). In addition, enzyme activity is used to determine in which of the tachyzoite and bradyzoite life cycle stages each pathway is operative Tachyzoites and bradyzoites are purified as described herein. The parasites are lysed in

50mM HEPES (pH7.4) containing 20% glycerol, 0.25% Triton X-100 and proteinase inhibitors (5mM PMSF, 5FM E64, 1FM pepstatin, 0.2mM 1,10-phenanthroline). This method has proven successful for measurement of phosphofructokinase, pyruvate kinase, lactate dehydrogenase, NAD- and NADH-linked isocitrate dehydrogenases and succinic dehydrogenase activity in tachyzoites and bradyzoites of *T. gondii* (Denton *et al.*, 1996a,b).

1) GSAT: GSAT activity is measured by the method of Jahn *et al.*, (1991), which uses GSA as substrate. GSA is synthesized according to methods of Gough *et al.* (1989). Heat-inactivated (60°C, 10') lysates are employed as non-enzymatic controls. ALA is quantified following chromatographic separation (Weinstein and Beale, 1985). This approach allows the definitive detection of GSAT activity in crude extracts.

2) ALA Synthase: To determine whether parasites contain ALA synthase, an activity also present in mammalian host cell mitochondria, cell fractions from purified parasites are assayed. (Weinstein and Beale, 1985) ALA produced from added glycine and succinyl CoA is quantified as for GSAT.

3) Isocitrate Lyase: The biochemical assay for isocitrate lyase activity used is the method of Kahn *et al.* (1977).

4) Alternative Oxidase: activity is measured in parasite lysates or purified mitochondria or plastids by oxygen uptake using an oxygen electrode described by Bass *et al.* (1990) Confirmation of the oxidation being due to alternative oxidase(s) is

achieved by successful inhibition of oxygen uptake in the presence of 0.5mM SHAM, but not in the presence of KCN.

5) Shikimate Pathway: The biochemical assay for EPSP synthase, chorismate synthase, chorismate lyase; activity in cellular lysates is conducted as described by Mousdale and Coggins (1985) and Nichols and Green (1992).

6) Branched Amino Acids: The biochemical assay for hydroxy acid synthase is as described.

7) Amylopectin Synthesis: The biochemical assays for starch synthase, Q enzymes, and UDP-glucose starch glycosyl transferase are as described.

8) Lipid Synthesis: Assays for lipid synthases are as described.

Some of the additional representative enzyme assays are precisely as described by Mousdale and Coggins(1985) and are as follows:

5-Enolpyruvylshikimate 3-phosphate synthase is assayed in forward and reverse directions as described previously (Mousdale and Coggins 1984).

Shikimate:NADP oxidoreductase (shikimate dehydrogenase), shikimate kinase, 3-Dehydroquinase (DHQase) are assayed. Assay mixtures contained in a total volume of 1 ml: 100 mM potassium phosphate (pH 7.0) and 0.8 mM ammonium 3-dehydroquinate. 3-Dehydroquinate synthase is assayed by coupling for forward reaction to the 3-dehydroquinase reaction; assay mixtures contained in a total volume of 1 ml: 10 mM potassium phosphate (pH 7.0), 50 μM NAD^+, 0.1 mM $CoCl_2$, 0.5 nkat partially-purified *Escherichia coli* DHQase

and (to initiate assay) 0.4 mM DAHP. The DAHP is prepared from *E. coli* strain AB2847A and DHQase from *E. coli* strain ATCC14948.

Assay of DAHP synthase is by a modification of the method of Sprinson et al.. Assay mixtures contained in a total volume of 0.5 ml: 50 mM 1,3-bis [tris(hydroxymethyl)-methylamino] propane-HCl (pH 7.4), 1 mM erythrose 4-phosphate, 2 mM phosphoenolpyruvate and 1 mM $CoCl_2$. The reaction is initiated by the addition of a 50 to 100 µl sample containing DAHP synthase and terminated after 10 min at 37°C by 100 µl 25% (w/v) trichloroacetic acid. The mixture was chilled for 1 h and centrifuged to remove precipitated protein. A 200 µl aliquot of the supernatant was mixed with 100 µl 0.2 M $NaIO_4$ in 9 M H_3PO_4 and incubated at 37°C for 10 min; 0.5 ml, 0.8 M $NaASO_2$ and 0.5 M Na_2SO_4 in 0.1 M H_2SO_4 in 0.1 m H_2SO_4 was then added and the mixture left at 37°C for 15 min; 3 ml 0.6% (w/v) sodium thiobarbiturate and 0.5 M Na_2SO_4 in 5 mM NaOH was added and the mixture placed in a boiling-water bath for 10 min. After cooling to room temperature the solution was centrifuged (8500 xg, 2 min) and the optical density at 549 nm read immediately. Appropriate controls assayed in triplicate lack substrates, sample or both."

Another representative assay is an assay for chorismate lyase which is as described by Nichols and Green, 1992:

Chorismate lyase assays are carried out in a volume of 0.5 ml containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM 2-mercaptoethanol, 60 µM chorismate, and 0.2 to 4 U of chorismate lyase After incubation at 37°C for

30 min, 4-hydroxybenzoate is detected and quantitated by high-pressure liquid chromatography (HPLC). Fifty microliters of each reaction mixture is applied to an HPLC system (Waters 625) equipped with a Nova-Pak C_{18} column equilibrated in 5% acetic acid and monitored at 240 nM. The height of the 4-hydroxybenzoate peak is compared with those of standard curves generated by treating known amounts of 4-hydroxybenzoate in a similar manner. One unit of chorismate lyase activity is defined as the amount of enzyme required to produce 1 nmol of 4-hydroxybenzoate in 30 min at 37°C.

Assays for 4-aminobenzoate and 4-amino-4-deoxychorismate are performed as described previously." Enzyme Assays: The 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase assay entailed monitoring the generation of EPSP using HPLC. Reaction components were separated using a Hypersil H3APS2 HPLC column (Hichrom Limited, Reading, UK) and a NaH_2PO_4 elution gradient (50-400 mM). UV spectra (200-320 nm) of the column eluate were collected to identify eluants. Shikimate-3-phosphate and 5-enolpyruvylshikimate-3-phosphate, synthesized enzymatically and purified to at least 95% purity as described (12), eluted after 3.9 and 6.8 min, respectively; phosphoenolpyruvate did not interfere with the EPSP detection and eluted after 5.3 min. The peaks at 215 nm were integrated; the EPSP produced was quantified using a standard curve of authentic EPSP. Parasite extracts were produced at 4°C by suspension of pure tachyzoites in extraction buffer (50 mM Tris HCl, pH 7.5, containing complete TM protease inhibitor cocktail [Boehringer Mannheim, 1 tablet per 50 ml buffer]), sonication 3 times for 3 seconds at 30 second

intervals, and centrifugation at 12000 g for 15 min. The resulting supernatant was diluted 6-fold with extraction buffer and loaded onto a ResourceQ column (1 ml, Pharmacia) equilibrated with extraction buffer. The bound protein was eluted in a single step using extraction buffer containing 500 mM KCl. The eluted material was used for enzyme assay. The assay mix contained 1 mM phosphoenolpyruvate, 1 mM SP and 50 mM HEPES, pH 7.5. The reaction was started by addition of parasite extract and incubation was at 30°C. Times 10 :l aliquots were subjected to HPLC analysis. Protein concentrations of lysates were determined using the Lowry method. (Roberts et al., 1998, In Press)

E. <u>Construction and Analysis of Gene "Knock-Outs"</u>

In order to determine whether a gene, *e.g.*, chorismate synthase or alternative oxidase is essential for growth or survival of the organism, gene knockout organisms are generated by the method of Roos *et al.*, 1996. Specifically, the strategy for creating mutants is with homologous recombination and to generate a targeted gene knock-out a sequential positive/negative selection procedure is used (Roos *et al.*, 1996). In this procedure positive and negative selectable markers are both introduced adjacent to, but not within the cloned and suitably mutated locus. This construct is transfected as a circular plasmid. Positive selection is applied to yield a single-site homologous recombinant that is distinguished from non-homologous recombinants by molecular screening. In the resulting 'pseudodiploid,' mutant and wild-type alleles flank selectable marker and other vector sequences In the next step, parasites are removed from positive selection, which permits recombination between the duplicated loci. This

event appears to occur at a frequency of 2×10^{-6} per cell generation. These recombinants are isolated with negative selection. Next, they are screened to distinguish those that have recombined in a manner that deletes the mutant locus and yields a wild-type revertant from those that deleted the wild-type gene to leave a perfect allelic replacement.

This 'hit-and-run' approach has the disadvantage of being time-consuming. Nonetheless, it offers several distinct advantages over other gene knock-out strategies. First, because gene replacement occurs by two sequential single-cross-overs instead of one double cross-over which is a very rare event, it is more likely to be successful. Second, because selectable marker(s) are located outside of the targeted gene itself, experiments are not limited to gene knock-outs. A variety of more subtle point mutations are introduced as allelic replacements. Third, this strategy provides a means of distinguishing essential genes from those which cannot be deleted for purely technical reasons. Specifically, if the hit-and-run mutagenesis procedure yields only wild-type revertants instead of the theoretical 1:1 ratio of wild-type:mutant, this provides positive evidence that the locus in question is essential.

An example is a knockout created for the chorismate synthase gene. It also can be made more general to include knockout of other genes for attenuated vaccines such as EPSP synthase and alternative oxidase. The parasite with the gene of interest to be knocked out is grown ("manufactured") *in vitro* in presence of product, but when used *in vivo* the needed product is not present The parasite functions as an attenuated vaccine as described below under vaccines A specific example follows: Specifically.

the strategy of product inhibition discussed above is also useful for growing gene knockout parasites (which lack a key gene for their survival) *in vitro* by providing the essential product and thus bypassing the need for the gene during *in vitro* propagation of the parasite. Such gene knockouts cultivated *in vitro* in this manner are useful attenuated organisms that are used as attenuated vaccines.

The chorismate synthase cDNA clones are used as hybridization probes for recovering genomic clones from a *T. gondii* genomic cosmid library. Coding regions are mapped onto the genomic clones using the cDNA clones as a guide. Appropriate sections are sequenced to verify the gene location. Ultimately, full genomic sequences are obtained. Enough of the genomic clones are sequenced to develop a strategy for generating a putative null allele. Segments that can be deleted at the 5' end of the coding region to generate an allele that is unlikely to generate a functional gene product are identified. A putative neutral allele is generated that can be distinguished from the wild type allele on the basis of an introduced restriction site polymorphism, but that does not differ in encoded protein sequence. These putative chorismate synthase-null and chorismate synthase-neutral alleles are cloned into the pminiHXGPRT transfection vector plasmid.

The resulting chorismate synthase -null and chorismate synthase-neutral plasmids are transfected into HXGPRT-negative strains of *T. gondii* (strains RH(EP)ʹHXGPRT [a ME49 derivative] Numerous independent clones are selected for survival on mycophenolic acid to select for insertion of the plasmid These strains are screened by Southern analysis designed to detect the presence of both the normal and

modified copies of the chorismate synthase gene and for tandem location of the two copies (with the vector HXGPRT gene between). This is the structure expected for insertion of the plasmid by homologous recombination at the *AroC* genomic locus (the "hit" needed for the hit-and-run gene knock-out strategy). The feasibility of recovering these strains is critically dependent upon the ratio of homologous to non-homologous integration following transfection, which will depend upon the length of homologous, genomic DNA in the clone (Donald and Roos, 1994; Roos *et al.*, 1996). Eight kb of homology is sufficient to obtain >50% homologous integration (Roos *et al.*, 1996).

HXGPRT clones with verified pseudodiploid structure of the chorismate synthase alleles are selected for loss of HXGPRT using 6-thioxanthine (the "run" part of the protocol). Numerous clones are selected. If the loss of HXGPRT is based upon random homologous exchange between the two chorismate synthase pseudodiploid alleles, theoretically half of the events should lead to excision of the modified chorismate synthase allele along with the HXGPRT, leaving the original wild type allele in the chromosome. The other half should excise the wild type allele, leaving the modified allele in the chromosome. During selection and grow-out of these clones, the medium is supplemented with chorismate at the concentration determined to best rescue cells from inhibitor toxicity. The purpose of the supplementation is to enhance the chances of recovering chorismate synthase-null strains. The genomic structure of the selected clones is examined by Southern analysis to confirm loss of the vector HXGPRT and of one copy of the chorismate synthase and to identify the remaining allele of chorismate synthase. The ratio of mutant to wild type is tabulated. The

chorismate synthase-neutral allele is intended as a positive control to confirm that either allele (wild type or mutant) can be lost in this procedure. If chorismate synthase-neutral strains can be recovered but chorismate synthase-null strains cannot, the conclusion is that the chorismate synthase gene is essential for growth. If it proves possible to recover chorismate synthase-null strains, they are subjected to further phenotypic analysis, first, using immunoblotting of electrophoretically separated cell extracts to confirm absence of chorismate synthase protein, then, determining if these strains show hypersensitivity to inhibitors of the alternative oxidase or to any of the other potential inhibitors. Sensitivity to chorismate synthase inhibitors is analyzed to determine the relative specificity of inhibition. If chorismate synthase is the sole target of the inhibitors, then the null mutants should be insensitive to further inhibition. Sensitivity analysis is conducted *in vitro* as described herein. Whether strains show alterations in expression of the alternative oxidase or in any stage-specific antigens is of interest. These analyses are conducted by immunoblotting of electrophoretically separated cell extracts. *In vivo* analysis using a mouse model is conducted to determine if these strains are infective and what stages of parasites can be detected following infection. Genetically altered *T. gondii* organisms are used to infect C3H/HeJ mice by the intraperitoneal route. Mortality is monitored and brains examined for cysts at 30 days post infection.

Knockouts with bradyzoite reporter genes are useful to determine whether these enzymes influence stage switch.

Stage switch also is characterized by quantitating relative amounts of parasite mRNA present in each stage of parasite using Northern blotting, isolation of mRNA and RT-PCR using a competitive inhibitor, and enzyme assay.

G. Reagents used for construction of "Knock-Outs"

Library

Me49 genomic libraries are used.

Plasmids

*p*miniHXGPRT. Contains *T. gondii* HXGPRT gene under control of DHFR-TS 5' and 3' flanking sequences. Functions as either a positive or negative selection marker (using 6-thioxanthine or mycophenolic acid, respectively) in suitable host strains.

Parasite Strains (obtained from AIDS Repository, Bethesda, Md.)

RH(EP). Wild-type host strain RH (highly pathogenic in mice).

RH(EP)'HXGPRT. HXGPRT knock-out mutant of RH strain. Suitable for positive selection of HXGPRT-containing vectors.

P(LK). Wild-type host strain P, (clonal isolate of strain ME49; produces brain cysts in mice).

P(LK)HXGPRT-. HXGPRT-deficient mutant of P strain. Suitable for positive selection of HXGPRT-containing vectors.

II. Antibodies

Antibodies have been raised against homologous plant enzymes by standard techniques for both polyclonal and monoclonal antibodies (Current Protocols in Immunology, 1996).

1) Heme Synthesis

Antibody to soybean, barley and synechococcus GSAT are polyclonal antibodies with preimmune sera the control for the barley and synechococcus antibodies.

2) Glyoxylate Cycle

T. gondii contains a glyoxylate cycle that allows growth using lipids as a carbon source, thus the lipid mobilization pathway of *T. gondii* is similar to the pathway of plants (Tolbert, 1980). A similar approach using polyclonal antibodies to isocitrate lyase and to malate synthase and preimmune control sera are used.

3) Alternative Energy Generation

Monoclonal and polyclonal antibodies to alternative oxidases in plants (McIntosh *et al.*, 1994) and *Trypanosomes* (Hill, 1976) are used.

4) Shikimate Pathway

To demonstrate that *T. gondii* has the same unique enzymes that permit interconversion of shikimate to chorismate as plants do, the antibody to shikimate pathway plant EPSP synthase is used.

5) Synthesis of Branched Chain Amino Acids

Antibodies to acetohydroxy acid synthase are used.

6) Amylose and Amylopectin Synthesis and Degradation

Antibodies to starch synthesis, branching (Q) enzymes and UDP glucose starch glycosyl transferase are used.

I. Complementation of Enzyme Deficient *E. coli* Demonstrates Functional Product

The *E. coli AroC* mutant which lacks chorismate synthase (*AroC*) was obtained from the *E. coli* genetic stock center. *AroC* bacteria is made competent to take up DNA by transformation with $CaCl_2$ treatment. Alternatively, the cells are electroporated to take up DNA. The presence of the plasmid is demonstrated in this system by growth on media which contains ampicillin, as the plasmid contains an ampicillin resistance gene. Complementation is confirmed by demonstrating growth on media lacking the product catalyzed by (*i.e.*, chorismate). Thus, this transformation/ complementation is used with the *T. gondii* cDNA library system or a construct which contains some or all of the chorismate synthase gene to transform the *AroC* mutant. Functional enzyme is then demonstrated.

J. Immunizations Of Mice For Polyclonal Antibody Production:

As an alternative approach if complementation studies are unsuccessful and the monoclonal antibodies to a plant protein are not cross reactive, purified plant protein is used to immunize mice to raise polyclonal antibodies to each enzyme. Where necessary, antibodies to the pertinent enzymes are generated in mice, ND4 outbred

mice are immunized with 20 μg of enzyme emulsified in Titermax complete adjuvant injected intramuscularly into their gluteal muscle. Two weeks later mice are immunized with a further 20 μg of enzyme emulsified in Titermax. After a further 2 weeks mice receive a further boost of enzyme alone in PBS by the intraperitoneal route. Mice are bled and the serum tested for specificity by the standard Western blotting technique.

K. Immunofluorescence

Antibodies used to identify enzymes in the Apicomplexan metabolic pathways disclosed here are used for immunofluorescence studies. Examples are demonstration of alternative oxidase in *T. gondii* by immunofluorescence assay (IFA). *T. gondii* alternative oxidase is immunolocalized to mitochondria.

L. ELISAs

ELISAs are used for documenting the presence and quantitating the amounts of alternative oxidase

M. Reporter Constructs To Demonstrate Organelle Targeting Are Made And Characterized As Described Using β Glucoronidase Or Other Chimeric Constructs

Importance of the targeting sequence for localization of the enzyme to an organelle is demonstrated with immunoelectronmicroscopy. Organelle targeting sequences in proteins expressed in bacteria which lack the organelle cause misfolding of proteins and thereby impair protein function

A useful reporter protein for a chimeric construct is β glucoronidase, expressed in *E. coli* under control of the 35S promoter of cauliflower mosaic virus The

glucoronidase alone without the transit sequence is expressed in parallel. The transit peptide construct is found in the plastid. The control glucoronidase is found in the cytoplasm. Antibodies to the chorismate synthase protein are also used to detect the presence of the product of the gene (with the transit sequence) in the plastid and the product of a construct (in which the transit sequence is not present) in the cytoplasm only. Further mutations and deletions are made which identify the minimal transit sequence using the same techniques as described above for the entire peptide. Antisense, ribozyme or intracellular antibodies directed against the transit sequence nucleic acid or translated protein are useful as medicines. The amino acid or nucleic acid which encodes the transit sequences are the bases for development of diagnostic reagents and vaccines.

N. Modifications of Inhibitory Compounds to Improve Oral Absorption Tissue Distribution (especially to brain and eye).

Tissue distribution is characterized using radiolabeled inhibitor administered to mice with its disposition to tissues measured. Compounds are modified to improve oral absorption and tissue distribution.

O. Methods to Demonstrate Protection Against Conjoint Infections

Infections are established and influence of an inhibitor or combination of inhibitors on outcomes are as outlined below.

Infections: Infections with *Toxoplasma gondii*, *Pneumocystis carinii*, *Mycobacterium tuberculosis*, *Mycobacterium avium* intracellular and *Cryptosporidium* parvum are

established alone and together using an immunosuppressed rodent model. Endpoints in these infections are:

Survival: Ability of an inhibitor to protect, measured as prolonged survival.

Parasitemia: This is measured using isolation of mRNA and RT-PCR with a competitive inhibitor for quantitation.

Tissue Parasite Burden: This is determined by quantitating brain and eye cyst numbers.

Inflammatory Response: This is noted in histopathologic preparations.

Representative combinations of inhibitors are NPMG and sulfadiazine, SHAM and atovaquone, NPMG and pyrimethamine, NPMG and SHAM.

P. Testing of Antimicrobial Compounds

Presence of inhibitory activity of new antimicrobial compounds is tested in enzymatic assays, *in vitro*, and *in vivo* assays as described above and in the literature.

Q. Efficacy, Safety, Pharmakokinetics, and Therapeutic/Toxic Index

The testing in murine models includes standard Thompson tests. Testing of antimicrobial agents for efficacy and safety in primate models for malaria is performed. Dosages are selected based on safety information available from data bases of information concerning herbicides and the literature. Measurements of serum and tissue levels of antimicrobial compounds are performed using assays which detect inhibitor concentrations and concentrations of their metabolites Representative assays are high performance liquid chromatography, and assaying tissues for percentage of radiolabeled compounds administered using liquid scintillation and other assays also are used.

R. Carcinogenicity and Teratogenicity

Standard assays to evaluate carcinogenicity include administration of medicines as described above to rodents and observation of offspring for teratogenic effects and carcinogenicity. Observation includes general physical examination, autopsy and histopathologic studies which detect any teratogenic or carcinogenic effects of medicines.

S. Constructs to Measure Parasitemia

Portions of genes are deleted and the shorter gene is used as an internal standard in RT PCR assays to measure amount of parasites present (Kirisits, Mui, Mack, McLeod, 1996).

T. Vaccine Constructs and Proteins and their Administration

These are prepared, and sensitivity and specificity are established as is standard in the literature and as described above. Tests and reagents include DNA constructs (Tine *et al.*, 1996) with the appropriate gene or portions of the gene alone or together, with adjuvants. Representative adjuvants include ISCOMS, nonionicsurfactant vesicles, cytokine genes in the constructs and other commonly used adjuvants. Native and recombinant proteins also are used in studies of vaccines. Protection is measured using immunologic *in vitro* assays, and by assessing survival and reduction of parasitemia and tissue parasite burden and prevention of congenital infection (McLeod *et al.*, 1988)

U. Preparation of Diagnostic Test Reagents and Diagnostic Tests:

These assays are as described (McLeod and Boyer, 1996). They include ELISAs in which antibodies to the proteins or peptides and recombinant proteins are used and PCR methodology in which primers to amplify DNA which encodes the enzymes or parts of this DNA are used. A test useful in an outpatient setting is based on conjugation of a monoclonal antibody to human red blood cells with antibody to peptides or proteins. The red cells are cross linked if the antibody to the parasite component interacts with the parasite component and agglutinates the red cells in the blood sample. ELISA and PCR can be utilized with samples collected on filter paper as is standard in Newborn Screening Programs and also facilitates outpatient and field use.

V. Antisense

Antisense oligonucleotides are short synthetic stretches of DNA and RNA designed to block the action of the specific genes described above, for example, chorismate synthase of *T. gondii* or *P. falciparum*, by binding to their RNA transcript. They turn off the genes by binding to stretches of their messenger RNA so that there is breakdown of the mRNA and no translation into protein. Antisense reagents have been found to be active against neoplasms, inflammatory disease of the bowel (Crohn's Disease) and HIV in early trials. Antisense oligonucleotides directed against the nucleic acids which encode the essential parasite metabolic process described herein are effective medicines to treat these infections. Antisense oligonucleotides also are directed against transit sequences in the genes. Antisense will not contain cytosine

nucleotides followed by guanines as this generates extreme immune responses (Roush, 1997). Antisense oligonucleotides with sequence for thymidine kinase also is used for regulatable gene therapy.

W. Ribozymes and Other Toxic Compounds

Ribozymes are RNA enzymes (Mack, McLeod, 1996) and they and toxic compounds such as ricins (Mahal et al, 1997) are conjugated to antisense oligonucleotides (see V, DNA), or intracellular antibodies (see X, for proteins), and these constructs destroy the enzyme.

X. Intracellular Antibodies

Intracellular antibodies are the Fab portions of monoclonal antibodies directed against the enzymes or portions of them (e.g., anti-transit sequence antibodies) which can be delivered either as proteins or as DNA constructs, as described under vaccines.

Y. Development of New Antimicrobial Compounds Based on Lead Compounds

The herbicide inhibitors comprise lead compounds and are modified as is standard. For example, side chain modifications or substitutions of groups are made to make more active inhibitors. Their mode of action and structure as well as the enzyme and substrate structures are useful in designing related compounds which better abrogate the function of the enzymes. Examples of such substrate or active site targeting are described above.

Native or recombinant protein is used in enzymatic assays and *in vitro* assays described above are used to test activity of the designed newly synthesized compounds. Subsequently, they will be tested in animals.

Z. Trials to Demonstrate Efficacy for Human Disease

Trials to demonstrate efficacy for human disease are performed when *in vitro* and murine and primate studies indicate highly likely efficacy and safety. They are standard Phase I (Safety), Phase II (small efficacy) and Phase III (larger efficacy with outcomes data) trials. For medicines effective against *T. gondii* tachyzoites, resolution of intracerebral *Toxoplasma* brain abscess in HIV-infected individuals with no other therapeutic options available due to major intolerance to available medicines is the initial strategy for Phase II trials. For medications effective against *T. gondii* bradyzoites, absence of development of toxoplasmic encephalitis in individuals with HIV infection and individuals who are seropositive for *T. gondii* infection followed after a one-month treatment for a 2 year period when their CD4 counts are low

Effective medicines demonstrate efficacy, as 50% of such individuals otherwise develop toxoplasmic encephalitis. When medications efficacious against bradyzoites and recrudescent toxoplasmic encephalitis in patients with AIDS are discovered and found to be safe, similar trials of efficacy and safety for individuals with recurrent toxoplasmic chorioretinitis are performed.

DEFINITIONS

3-deoxy-d-arabino-heptuloonate 7 phosphate synthase: An enzyme which functions in chorismate synthesis.

3-enolpyruvyshikimate phosphate synthase (3-phosphoshikimate-1-carboxyvinyltransferase): An enzyme which functions in chorismate synthesis.

3-NPA: An inhibitor of isocitrate lyase in the glyoxylate pathway and also of succinate dehydrogenase.

3-oxtaprenyl-4-hydroxybenzoate carboxylyase: An enzyme which functions in ubiquinone synthesis.

4-hydroxybenzoate octaprenyltransferase: An enzyme which functions in ubiquinone synthesis.

8-OH-quinoline: An inhibitor of the alternative oxidase.

Abscissic Acid Metabolism in Plants: A 15-carbon sequiterpenoid synthesized partly in plastids by the mevalonic acid pathway. Abscissic acid protects plants against stress and is a marker of the plant's maturation and activation of transcription, and causes dormancy. Inhibits protein synthesis and leads to specific activation and deactivation of genes.

Acetohydroxy acid synthase: Enzyme which catalyzes production of acetohydroxy acids (the branched chain amino acids valine, leucine and isoleucine in plants).

Alternative oxidase: An enzyme important in the alternative pathway of respiration. There are examples of alternative oxidases in plants and trypanosomes (Pollakis *et al.*, 1995; Rhoads & McIntosh, 1992; Clarkson *et al.*, 1989)

Alternative respiration or energy generation: A different pathway for energy generation utilizing the alternative oxidase and election flow in the electron transport chain which is not dependent on conventional cytochromes or heme.

Altered gene includes knockouts.

Amide: The R portion of the amino group has an amino group connected to a carbonyl carbon. Glutamine and asparagine are amides. Important for nitrogen transport and storage.

Amylopectin: A branched starch of plants. Also found in *T gondii* bradyzoites.

Amyloplast: Storage granule for starch in plants. Derived from chloroplasts.

Amylose: An unbranched starch of plants.

Anabolism: Formation of large molecules such as starch, cellulose, proteins, fats and nucleic acids from small molecules. Requires input of energy.

Anthranilate phosporibolsyltransferase: An enzyme which functions in tryptophan synthesis.

.Anthranilate synthase component I: An enzyme which functions in tryptophan synthesis.

Anthranilate synthase component II: An enzyme which functions in tryptophan synthesis.

Antimicrobial agent: A chemical, for example a protein or antisense nucleic acid which effectively inhibits or kills a pathogenic microbe. There are examples (Schwab *et al.*, 1994, Strath *et al.*, 1993; Beckers *et al.*, 1995, Blais *et al.*, 1993; Fichera *et al.*,

1995; Pfefferkorn & Borotz, 1994; Pfefferkorn *et al.*, 1992; Pukivittaykamee *et al.*, 1994).

Apicomplex: The common feature of Apicomplexan parasites including a conoid and rhoptry organelles and micronemes at the apical end of the parasite.

Apicomplexan parasite: A microorganism that belongs to the Apicomplexan group of parasites. These parasites share a number of morphologic features, including a conoid and rhoptry which are organelles in the cytoplasm at the apical end of the organism and plastids which are multilamellar structures. Representative examples of Apicomplexan parasites include *Toxoplasma gondii*, *Plasmodium*, *Cryptosporidia* and *Eimeria*.

Aromatic acid aminotransferase (aromatic transaminase): An enzyme which functions in tyrosine synthesis.

Aspartate, glutamate and glutamine synthesis: Involve glutamine synthase and glutamate synthetase and are plastid associated in plants. Glutamine synthase in plants is inhibited by the herbicide glufosinate (2 amino-4-[hydroxymethylphosphinyl) butanoic acid. Glutamine synthase also is present in animals.

ATP-phosphofructokinase: (ATP-PFK) May exert control over glycolytic pathway because a step when hexoses phosphate cannot also be used to form sucrose or starch. Nearly all animals lack PPi-PFK with plant-like substrate specificity (i.e. PPi, not ATP).

Auxins: Growth regulators in plants, which are tryptophan derivatives. Herbicides modeled on auxins are structural mimics of these compounds rather than inhibitors of auxin function

Biochemical pathways: Biochemical pathways include metabolic pathways. Any chemical reaction in life. Herein "biochemical pathways" and "metabolic pathways" are used interchangeably.

Bradyzoite: The slowly replicating life cycle stage of the Apicomplexan parasite *Toxoplasma gondii*. This stage is responsible for latent and recrudescent infection due to this parasite. The morphologic features which characterize this parasite stage are electron dense rhoptries and amylopectin granules. Bradyzoites contain a plastid organelle as do other life cycle stages of this parasite. This parasite stage also has specific antigens which other life cycle stages do not have, including bradyzoite surface antigen 4 and bradyzoite antigen 5 (lactate dehydrogenase), which is an intracellular and cyst matrix antigen. Bradyzoites exist together in a structure called a cyst which has a cyst wall and matrix. Cysts contain a few to thousands of bradyzoites. The cyst containing bradyzoites is a major means of transmission of the organism *Toxoplasma gondii* when it is ingested in meat which is not cooked to well done. It is also a form of the organism responsible for recrudescent eye and brain disease in infants and children who are congenitally infected with the parasite and also in patients whose immune system is not normal.

Branched chain amino acid synthesis (valine, leucine and isoleucine) involving acetohydroxy acid synthase, is the first of the series of reactions, is another metabolic pathway present in plants but not in animals.

Branched chain amino acids: Amino acids (valine, leucine and isoleucine), the synthesis of which can be inhibited by sulfonylurea and imidazolinone herbicides

There are examples in plants (Kuriki *et al.*, 1996; Morell *et al.*, 1997; Kortostee *et al.*, 1996; Grula *et al*, 1995; Khoshnoodi *et al.*, 1996).

Branching or Q enzyme: Forms branches in amylopectins between C6 of the main chain and C1 of the branch chain.

Catabolism: Degradation or breakdown of large molecules to small molecules, often releasing energy.

Calmodulin: is a calcium binding protein (Robson *et al.*, 1993)

Catechol 1,2-deoxygenase (phenol hydroxylase): An enzyme which functions in phenylalanine synthesis.

Chloroplast: A DNA-containing multilamellar organelle of plants and algae associated with metabolic pathways important for photosynthesis and other energy production. Chloroplasts utilize proteins encoded in their own DNA and also proteins encoded by nuclear DNA.

Chorismate: The product of the action of the enzyme EPSP synthase on shikimate.

Chorismate lyase: An enzyme responsible for the conversion of chorismate to 3,4-dihydroxybenzoate.

Chorismate mutase (7-phospho-2-dehydro-3-deoxy-arabino-heptulate-aldolase): An enzyme which functions in chorismate synthesis.

Chorismate synthase: An enzyme responsible for the conversion of 3-phospho 5-enolpyruvyl shikimate to chorismate.

Chorismate: The product of the action of the enzyme EPSP synthase on shikimate.

Competitive inhibitors: Structures sufficiently similar to the substrate that they compete for the active site of the enzyme. Addition of more natural substrate overcomes effect of the inhibitor.

Components: includes nucleic acids, proteins, peptides, enzymes, peptide targeting sequences, transit peptides, carbohydrates, starch, lipids, hormones, for example those listed in Table 1 and other constituents of metabolic pathways or products derived from these components.

Conventional energy generation: Usual pathways of generation of energy in mitochondria utilizing cytochromes for the transfer of electrons.

Conversion of Fats to Sugars in Plants: Occurs by oxidation and the glyoxylate cycle.

Cryptosporidiosis: The disease due to the Apicomplexan parasite *Cryptosporidium parvum*. It causes self-limited diarrhea or no symptoms in immunologically normal individuals. In individuals who have immunocompromising illnesses, such as the acquired immune deficiency syndrome, Cryptosporidiosis causes life-threatening, persistent, copious, watery diarrhea.

Cryptosporidium parvum: *Cryptosporidium parvum* is an Apicomplexan parasite which causes cryptosporidiosis.

Cyanide-insensitive, non-heme "alternative" oxidase is a metabolic activity that is found in most eukaryotic plants and algae and is absent from multicellular animals. The alternative oxidase is a single polypeptide enzyme that lacks heme and can serve as the terminal electron acceptor to support respiratory growth of *E. coli* in the absence of

heme. The coupling efficiency of this oxidase is lower than that of the cyanide-sensitive cytochrome oxidase. That is, not as many protons are pumped across the mitochondrial inner membrane in parallel with electron transfer through the alternative oxidase as they are through the cytochrome oxidase. The alternative oxidase appears to be used by plants and algae only under certain conditions. The alternative oxidase also is used during different life-cycle stages or under different environmental conditions. Thus, inhibitors of the alternative oxidase may act cooperatively or synergistically with GSAT inhibitors.

Cyclohexadienyl dehydratase: An enzyme which functions in phenylalanine synthesis.

Cyclohexadienyl dehydrogenase: An enzyme which functions in tyrosine synthesis.

Cytochrome oxidase: An enzyme utilized in the conventional pathway of energy generation.

Dehydroquinate dehydratase: An enzyme which functions in chorismate synthesis.

Deoxyribonucleases: Enzymes which are hydrolases which hydrolyze DNA (phosphate esters)

***Eimeria bovis*:** Causes bovine eimeriosis.

Eimeria maxima and Eimeria tenella: Cause eimeriosis in chickens.

***Eimeria*:** A group of Apicomplexan parasites which cause gastrointestinal disease in agriculturally important animals including poultry and cattle. These economically important parasites include *Eimeria tenella*, *E. maxima* and *E. bovis*

Endosymbiont: An organism which is taken up by another organism and then lives within it.

Enzyme: A protein which catalyzes (makes more rapid) the conversion of a substrate into a product. Enzymes are catalysts which speed reaction rates generally by factors between 10^8 and 10^{20}. They may require ion or protein cofactors. Control is by products and environmental changes. There are more than 5000 enzymes in living systems. Enzymes are named with common or trivial names, and the suffix-ase which characterizes the substrate acted upon (e.g., cytochrome oxidase removes an electron from a cytochrome). Sequential series of steps in a metabolic pathway. Enzymes that govern the steps in a metabolic pathway are sometimes arranged so that a kind of assembly-line production process occurs.

EPSP synthase: An enzyme important in the conversion of shikimate to chorismate.

EST: Expressed sequence tag; a short, single pass cDNA sequence generated from randomly selected library clones.

Eukaryote: Microorganism or phylogenetically higher organism, the cells of which have a nucleus with a limiting membrane.

Fatty Acid Synthesis in Plants: Occurs in chloroplasts of leaves and proplastids of seeds and roots. Mainly palmitic acid and oleic acid. AcetylCo A carboxylases differ in plants and animals. Linoleic acid synthase and linoleneic acid synthase are lipid synthases present in plants and not animals

Glycolysis → pyruvate →acetyl CoA

Example:

8 acetyl CoA +7 ATP^{3-} + 14 NADPH + 1+H^{+}→ palmityl CoA + 7 CoA + 7ADP^{2-} + 7H_2PO4 + 14 $NADP^{+}$ + 7H_2O.

Fragment: Refers to a sequence of nucleic acids or aminoacids, where a fragment is sufficient to function as a component of or product derived from an Apicomplexan as defined herein.

Gabaculine: An inhibitor of the enzyme GSAT in the heme synthesis pathway.

Gene: Nucleotide sequence which encodes an amino acid sequence or another nucleotide sequence.

Giberellin Metabolism in Plants: Plant hormones which promote plant growth, overcome dormancy, stimulate G1 to S transition and shorten S phase of cell cycle, increase hydrolysis of starch and sucrose into glucose and fructose. They are derivatives of ent-gibberellane skeleton synthesized from a 2acetyl CoA to mevalonic acid to isopenternyl pyrophosphate to 4 isopentenyl pyrophosphate to geranylgeranyl pyrophosphate to copalylpyrophosphate to kaurene to kaurenol to keaurenal to kaurenoic acid to GA_{12} aldehyde to other giberellins. These functions are not clearly established but it is hypothesized that hydrolysis of starch to sugar occurs by inducing formation of amylase enzymes. Isoprenoid compounds, diterpenes synthesized from acetate units of acetyl coenzyme A by mevalonic acid pathway stimulate growth. Inhibitors of giberellin synthesis include phosphon D. Amo 1618 (blocks conversion of geranyl pyrophosphate to CO palylpyrophosphate). phosphon D, which also inhibits conversion of (oxidation) formation of Kaurene. CCC or cycocel, ancymidol, and

pactobutrazol (blocks oxidation of karene and kaurenoic acid). Young leaves are major sites for giberellin synthesis. These plant hormones which induce hydrolysis of polysaccharide into hexoses are used in glycolysis. When hexoses are abundant, glycolysis is more rapid.

Glutamyl-tRNA reductase: An enzyme which functions in heme synthesis.

Glutamyl-tRNA synthetase: An enzyme which functions in heme synthesis.

Glycolysis in Plants: Several reactions of glycolysis also occur in plastids. Glycolysis = lysis of sugar; degradation of hexosis to pyruvic acid in plants. In animals, degradation of glycogen (animal starch) to pyruvate. Plants form no glycogen.

Glyoxylate pathway: The pathway important for lipid degradation which takes acetyl CoA and converts it to CoA-SH through the conversion of isocitrate to C4 acids including succinate. This pathway utilizes isocitrate lyase and also converts glyoxylate to malate, a reaction catalyzed by the enzyme malate synthase. The glyoxysome or Glyoxylate pathway which is cytoplasmic in certain algae involves isocitrate lyase and malate synthase to metabolize lipids and provide C4 acids. A metabolic distinction between autotrophic eukaryotes and heterotrophs is the presence of a glyoxylate cycle. This cycle employs two enzymes, isocitrate lyase and malate synthase, to bypass the two decarboxylation steps of the TCA cycle and enables the utilization of carbon stored in fatty acids for growth. In plants, the enzymes of the glyoxylate cycle are compartmentalized within a unique single-membrane-bound organelle, the glyoxysome. In certain algae, the cycle is entirely cytoplasmic In plants, these enzymes are most

abundant during germination and senescence. In animals, the glyoxylate cycle enzymes have been described as being present only during starvation.

Glyoxysome: An organelle which in some instances contains enzymes important in the glyoxylate cycle.

GSAT: Glutamate-l semialdehyde aminotransferase is the enzyme important in heme synthesis for the conversion of glutamate semialdehyde to ALA (δ-aminolevulinic acid).

Heme synthesis pathway: A metabolic pathway important for generation of heme, porphyrins and other iron sulfated proteins used in mitochondria in the conventional pathway of energy generation. This pathway occurs in plant chloroplasts and uses the nuclear encoded enzyme GSAT. A metabolic distinction between plants and animals occurs in the heme biosynthesis pathway. Non-photosynthetic eukaryotes, including animals, yeast, fungi and protists, produce δ-aminolevulinic acid (ALA), the common precursor of heme biosynthesis, by condensation of glycine and succinate. In contrast, photosynthetic organisms, including plants, algae and cyanobacteria, *E. coli* and some other bacteria synthesize ALA from glutamate (a 5-carbon pathway). *Euglena* utilize both condensation of glycine and succinate and the 5 carbon pathway to produce δ-aminolevulinic acid. *T. gondii* also has the ALA synthase which results in formation of heme by condensation of glycine and succinate, as does *P. falciparum* (Surolia and Padmanaban, 1992) Expression of this enzyme is developmentally regulated. For example, in plants, GSAT is most abundant in the leaves There are examples in plants (Matters & Beale, 1995, Elich *et al.*, 1988)

Herbicide: A compound which kills plants or algae

Hydrolases: Enzymes which break chemical bonds (e.g., amides, esters, glycosides) by adding the elements of water.

Imidazolinones: Inhibitor of acetohydroxy acid synthase (an enzyme involved in the synthesis of branched chain amino acids, a pathway not in or rarely present in animals,

Indole-3-glycerol phosphate synthase (anthranilateisomerase), (indoleglycerol phosphate synthase): An enzyme which functions in tryptophan synthesis.

Inhibitor: A compound which abrogates the effect of another compound.

A compound which inhibits the replication or survival of a microorganism or the function of an enzyme or key component of a metabolic pathway or otherwise abrogates the function of another key molecule in a microorganism or other organisms or plant.

Isocitrate lyase: An enzyme which functions in glyoxylate cycle.

Isomerases: Enzymes which rearrange atoms of a molecule to form a structural isomer.

Isoprenoid Metabolism in Plants: Terpenes are isoprenoids that lack oxygen and are pure hydrocarbons; 5 carbon units with some of the general properties of lipids. Gibercllins and abscidic acid are others of this vast complex of compounds not found in animals.

Isoprene units (head) are CH_2 - CH3C = CH - CH_2 (tail) and are synthesized entirely from acetate of acetyl CoA and restricted to plants. Synthesized by mevalonic acid pathway because mevalonate is an important intermediate.

Kinases: A subclass of transferases which transfer phosphate groups, especially from ATP.

Latency: The dormant form of the parasitic infection. One example is with *Toxoplasma gondii* in which the infection is not active and the parasite is primarily within cysts in the bradyzoite phase of the life cycle. Another example is the hypnozoite phase of *Plasmodium falciparum*.

Ligases or Synthetases: Enzymes which join two molecules coupled with hydrolysis of ATP or other nucleoside triphosphate.

Lipases: Enzymes which are hydrolases which hydrolyze fats (esters)

Lipid and terpene synthesis associated with plant plastids. Also see fatty acid synthesis and terpenes.

Lysases: Enzymes which form double bonds by elimination of a chemical group.

Malaria: Disease due to pathogenic *Plasmodia*. Examples are *Plasmodium falciparum*, *Plasmodium virax*, *Plasmodium ovale*, *Plasmodium malaria*, in humans and *Plasmodium knowlesii* in monkeys.

Malate synthase: An enzyme which functions in glyoxylate cycle.

Metabolic pathways: Both anabolism and catabolism consist of metabolic pathways in which an initial Compound A is converted to another B, then B is converted to C, C to D and so on until a final product is formed. In respiration, glucose is the initial compound, and CO_2 and H_2O are the final products. There are approximately 50 distinct reactions in respiration but other metabolic pathways have fewer reactions

Herein the phrases "metabolic pathways" and "biochemical pathways" are used interchangeably.

Metabolism: Chemical reactions that make life possible. Thousands of such reactions occur constantly in each cell.

Microbes: Organisms which are visible only with use of a microscope. Some cause disease (are pathogenic).

Microbicidal: An agent (*e.g.*, an antibiotic or antimicrobial compound) which kills microbes.

Mitochondria: An organelle responsible for the generation of energy.

Multilamellar: An adjective which refers to the multiple membranes within an organelle.

Noncompetitive inhibitors: Combine with enzymes at sites other than active site.

"Not involve": Are not a starting point, a component, or a product of the metabolic pathways described in relation to this invention.

NPMG: An inhibitor of EPSP synthase in the shikimate pathway.

Nucleic Acid: Deoxyribonucleic acid and ribonucleic acid molecules are constructed of a sugar phosphate backbone and nitrogen bases; important in the encoding, transcription and synthesis of proteins.

Oocyst: A life cycle stage of a parasite, *e.g.*, *Toxoplasma gondii* that contains sporozoites *T. gondii* sporozoites and oocysts form only in the cat intestine. This form of the parasite is able to persist in nature in warm, moist soil for up to a year and is highly infectious. Sporulation occurs several days after excretion of oocysts by

members of the cat family (*e.g.*, domestic cats or wild cats such as lions or tigers). Sporulation must occur before the oocyst becomes infectious.

Organelle: A structure within a cell. Examples are plastids, mitochondria, rhoptries, dense granules and micronemes.

Oxidoreductases (oxidases, reductases, dehydrogenases): Remove and add electrons or electrons and hydrogen. Oxidases transfer electrons or hydrogen to O_2 only.

Paraminobenzoic acid (PABA): A product of the shikimate pathway in plants.

Parasite: An organism which lives in or on a host for a period of time during at least one life-cycle stage.

Phagemid: Plasmid packaged within a filamentous phage particle.

Phosphoribosyl anthranilate isomerase: An enzyme which functions in tryptophan synthesis.

Plant-like: Present in algae and higher plants, but not or only rarely, or in unusual circumstances in animals.

***Plasmodium falciparum*:** One species of *Plasmodium* which causes substantial human disease.

***Plasmodium knowlesii*:** A species of *Plasmodium* which causes malaria in monkeys.

Plastid: A multilamellar organelle of plants, algae and Apicomplexan parasites which contains its own DNA separate from nuclear DNA. Plastids have been described in studies of Apicomplexan parasites which used electron micrographs (Siddall, 1992,

Williamson *et al.*, 1994; Wilson *et al.*, 1991; Wilson *et al.*, 1994; Wilson *et al.*, 1996; Hackstein *et al.*, 1995; McFadden *et al.*, 1996).

Polymerases: Enzymes which link subunits (monomers) into a polymer such as RNA or DNA.

PPi phosphofructokinase Type I : An enzyme present in plants that functions in glycolysis and in a number of organisms regulates glycolysis. In plants and protozoans PPi, not ATP (as in animals) is utilized to synthesize Fru-1-6P_2 from Fru 6P. Activity is not stimulated in protozoa by Fru-2-6-P_2 (Peng & Mansour, 1992; Denton et al., 1996a,b).

Prephenate dehydratase (phenol 2-monoxygenase): An enzyme which functions in phenylalanine synthesis.

Prephenate dehydrogenase: An enzyme which functions in tyrosine synthesis.

Product: The end result of the action of an enzyme on a substrate.

Prosthetic group: Smaller organic nonprotein portion of an enzyme essential for catalytic activity. Flavin is an example.

Proteinases: Enzymes which are hydrolases which hydrolyze proteins (peptide bonds).

PS II: Important alternative means for producing energy within chloroplasts and apparently also described as being present in Apicomplexans.

Pyrimethamine: An inhibitor of the conversion of folate to folinic acid and thus an inhibitor of nucleic acids production effective against *Toxoplasma gondii*.

Recrudescence: Reactivation of the parasite *Toxoplasma gondii* from its latent phase

Respiration: Major catabolic process that releases energy in all cells. It involves breakdown of sugars to CO_2 and H_2O.

Ribonucleases: Enzymes which are hydrolases which hydrolyze RNA (phosphate esters).

Salicylic Acid Metabolism in Plants: Salicylic acid is a plant hormone which promotes activity of cyanide resistant respiration.

SHAM: An inhibitor of the alternative oxidase.

Shikimate dehydrogenase: An enzyme which functions in chorismate synthesis.

Shikimate kinase: (shikimate 3-phosphotransferase) An enzyme which functions in chorismate synthesis.

Shikimate pathway A pathway that involves the conversion of shikimate to chorismate and subsequently the production of folate, aromatic amino acids, and ubiquinone. This pathway contains enzymes which lead to production of folic acid, ubiquinone, and aromatic amino acids. Folate, ubiquinone, and aromatic amino acids are products derived from this pathway in plants. There is sequential use of products of these pathways as reactants in subsequent enzymatically catalyzed reactions. For example, ubiquinone is an essential coenzyme for both conventional and alternative respiration. There are examples in plants, bacteria and fungi. (Bornemann *et al.*, 1995; Marzabadi *et al.*, 1996; Ozenberger *et al.*, 1989; Shah *et al.*, 1997; Gilchrist & Kosuge, 1980; Walsh *et al.*, 1990; Weische & Leisterner, 1985; Green *et al.*, 1992; Young *et al.*, 1971)

Shikimate: The substrate for EPSP synthase

Sporozoite: Another phase of the life cycle of *Toxoplasma gondii* which forms within the oocyst which is produced only within the cat's intestine. A highly infectious form of the parasite.

Stage specific: A characteristic of the parasite which is expressed or present only in a single life cycle stage or in some but not all life cycle stages.

Starch Degradation in Plants: 3 enzymes: α amylase (attack 1, 4 bonds of amylopectin (to maltose) and amylase (to dextrin). Many activated by Ca++. Located in chloroplasts. β amylase hydrolyzes starch to maltose; starch phosphorylase degrades starch beginning at nonreducing end. (Starch + H2PO4 *⇌ glucose + - Phosphate) Only partially degrades amylopectin debranching enzymes hydroxy 1.6 branch linkage in amylopectin. Hexoses cannot move out of chloroplasts or amyloplasts thus must be converted to triose phosphate (3-PG aldehyde and dehydroxyacetone P). sucrose + UDP *⇌ fructose + UDP-glucose, *= sucrose synthase

Starch Formation in Plants: Animals store starch as glycogen and plants store starch as amylose and amylopectin. Starch synthesis is dependent on starch synthase and branching Q enzymes. Mutations in genes encoding these enzymes lead to diminished production of starch. In addition, amylopectin synthesis predominates in plant mutants without UDP-glucose-starch glycosyl transferase whereas wild type plants with this enzyme make predominantly amylose and a smaller amount of amylopectin. In the mutant UDP-glucose-starch glycosyl transferase appears to be transcriptionally regulated Amino acid motifs that target proteins to plant plastid organelles have been

identified in UDP-glucose starch glycosyl transferase, as have other motifs that determine transit into plastids and mitochondria and these have been used to target the transported proteins in plants. Reactions include: ADPG + small amylose (in glucose) *→larger amylose (N+1 glucose units)+ADP.*= starch synthase K+. Branching or Q enzymes form branches in amylopectins between C6 of the main chain and C1 of the branch chain. There are examples in plants (Abel *et al.*, 1996; Van der Leif *et al.*, 1991; Van der Steege *et al.*, 1992).

Starch synthase: catalyzes reaction: ADPG + small amylose (n-glucose units) → larger amylose n+1 glucose units + ADP and is activated by K+. Thus, sugars not starch accumulate in plants deficient in K+.

Starch: Major storage carbohydrate of plants, used for energy regeneration. Two types composed of D glucose connected by 1. 4 bonds which cause starch chains to coil into helices. The two types are amylose and amylopectin. Amylopectin is highly branched with the branches occurring between C-6 of a glucose in the main chain and C-1 of the first glucose in the branch chain (-1.6 bonds). Amyloses are smaller and have fewer branches. Amylopectin becomes purple or blue when stained with iodine-potassium-iodine solution. Amylopectin exhibits a purple red color. Control of starch formation is by K+ and a light activated sucrose phosphate synthase enzyme, invertase enzymes and the allosteric effect of fructose 2. 6 phiphosphate adenosine diphosphoglucose (ADPG) donates glucoses to form starch. Starch in amyloplasts is a principal respiratory substrate for storage organs

Substrate reactant: Enzyme substrates have virtually identical functional groups that are capable of reacting. Specificity results from enzyme substrate combinations similar to a lock and key arrangement.

Substrate: The protein on which an enzyme acts that leads to the generation of a product.

Sucrose Formation Reactions in Plants: UTP+glucose 1 phosphate $\rightleftharpoons$UDPG+PPi

PPi+$H_2$0 +2 Pi

UDPG+fructose 6 phosphate$\rightleftharpoons$sucrose-6-phosphate+UDP

Sucrose-6-PHOSPHATE+$H_2$0$\rightarrow$sucrose+Pi

UDP+ATP$\rightleftharpoons$UTP+ADP

$\therefore$glucose-1-phosphate+fructose 6 phosphate+2 $H_2$0+ATP$\rightarrow$sucrose 3Pi+ADP

Sulfadiazine: An antimicrobial agent effective against *Toxoplasma gondii* which competes with para-aminobenzoic acid important in folate synthesis.

Sulfonylureas: Inhibitors of acetohydroxy acid synthase (an enzyme involved in the synthesis of branched chain amino acids, a pathway not or rarely present in animals).

Synergy: The effect of a plurality of inhibitors or antimicrobial agents which is greater than the additive effect would be combining effects of either used alone. Synergy occurs particularly when the action of an enzyme (which is inhibited) on a substrate leads to a product which is then the substrate for another enzyme which also is inhibited; that is, when the enzymes are in series or follow one another in a pathway. This effect occurs because the production of the first enzymatic reaction provides less substrate for the second reaction and thus amplifies the effect of the second inhibitor or

antimicrobial agent. In contrast, an additive effect is when the effect of the compounds used together is simply the sum of the effects of each inhibitory compound used alone. This most often occurs when the pathways are in parallel, for example, when the effect on the first enzyme does not modify the effect of the second enzyme.

Tachyzoite: The rapidly replicating form of the parasite *Toxoplasma gondii*.

***Theileria*:** An Apicomplexan parasite infecting cattle.

***Toxoplasma gondii*:** A 3-5 micron, obligate, intracellular, protozoan parasite which is an Apicomplexan.

Toxoplasmosis: Disease due to *Toxoplasma gondii*.

Transit (translocation) peptide sequence: Amino acid sequence which results in transit into or out of an organelle. These have been described in plants (Volkner & Schatz, 1997; Theg & Scott, 1993). Herein we also call it a "metabolic pathway," although it is part of a component of a metabolic pathway or may function independently of a metabolic pathway.

Triazine: An inhibitor of PS II complex.

Tryptophan synthase alpha subunit: An enzyme which functions in tryptophan synthesis.

Tryptophan synthase beta subunit: An enzyme which functions in tryptophan synthesis.

Type I PPi phosphofructokinase is another enzyme present in plants and there is different substrate utilization by phosphofructokinases of animals

UDP glucose starch glycosyl transferase: An enzyme involved in production of amylose in plants. The absence of this enzyme leads to starch formation as amylopectin rather than amylose.

USPA: Gene which encodes a universal stress protein. This has been described in *E. coli* (Nystrom & Neidhardt, 1992).

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WE CLAIM:

1. A pharmaceutical composition having a parasite with a chorismate synthase gene that is knocked out.

2. An immunogenic composition comprising an attenuated parasite of claim 1.

3. The parasite of claim 1 is *T. gondii*.

4 An immunogenic composition comprising a cDNA molecule encoding chorismate synthase, said molecule complementary to an mRNA from *T. gondii*.

5. An assay for a candidate inhibitor of *T. gondii*, said assay comprising:

(a) adding a chorismate synthase - green fluorescent reporter protein construct to a parasite of the *T. gondii* species;

(b) contacting the parasite with the candidate inhibitor;

(c) comparing the amounts of green fluorescent reporter protein in the parasite in the presence and absence of the candidate inhibitor; and

(d) inferring that the candidate inhibitor is an inhibitor of the parasite if there is significantly less reporter protein when the candidate inhibitor is present.

6. A method for detecting a life cycle stage in a sample tested for *T. gondii* said method comprising:

(a) determining an amount of chorismate synthase present in the sample; and

(b) comparing the amount to amounts of standards determined from known life cycle stages.

7. The method of claim 6, wherein the sample is derived from a cat.

Fig. 1A

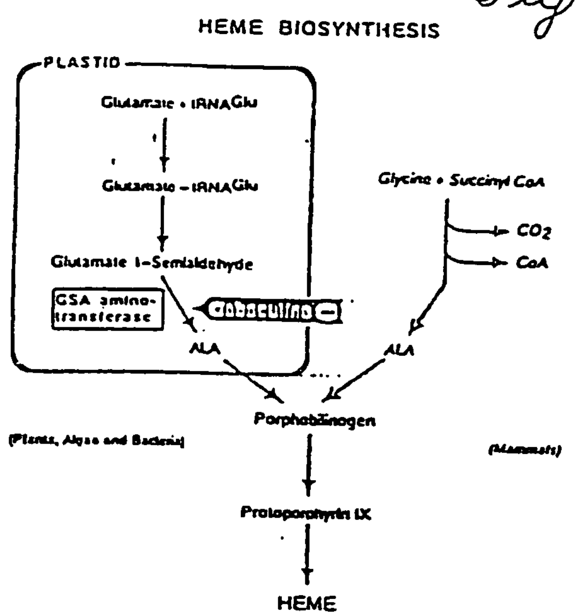
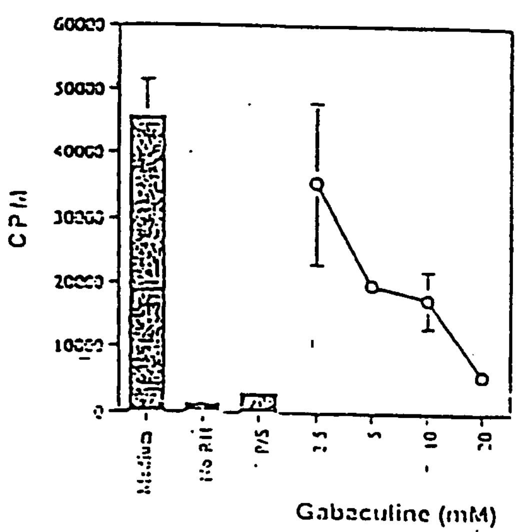


Fig. 1B



RECTIFIED SHEET (RULE 91)
ISA/EP

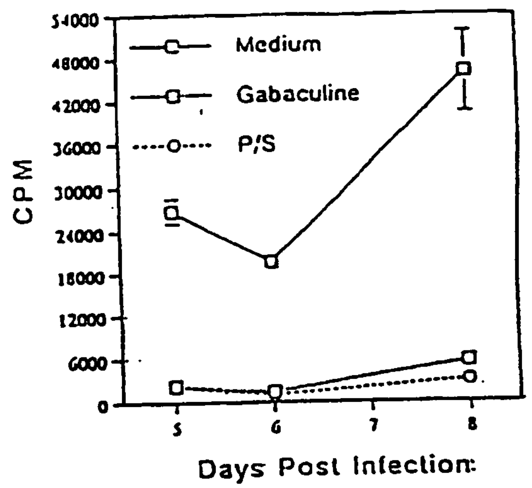

CPM
54000
48000
42000
36000
30000
24000
18000
12000
6000
0
Medium
Gabaculine
P/S
5
6
7
8
Days Post Infection

Fig. 1c

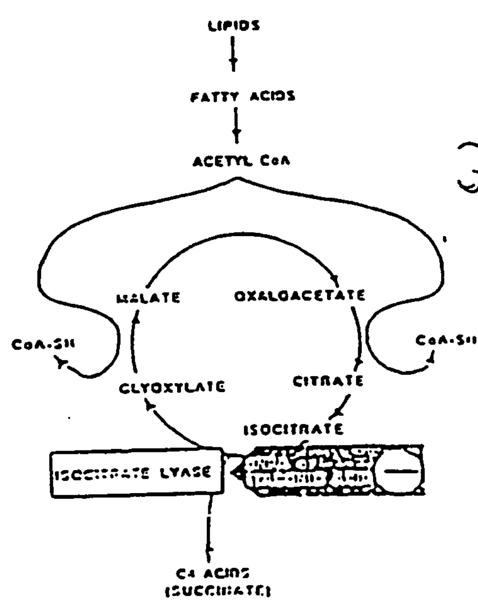

LIPIDS
FATTY ACIDS
ACETYL CoA
MALATE
OXALOACETATE
CoA-SH
CoA-SH
GLYOXYLATE
CITRATE
ISOCITRATE
ISOCITRATE LYASE
C4 ACIDS
(SUCCINATE)

Fig. 2A

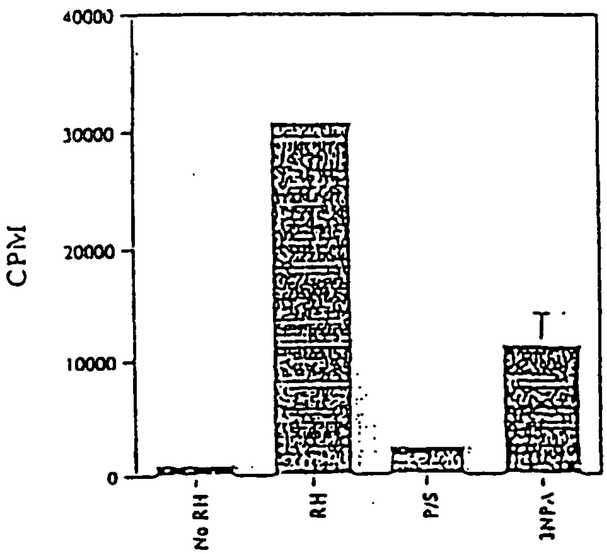


Fig. 2B

Mitochondrion*

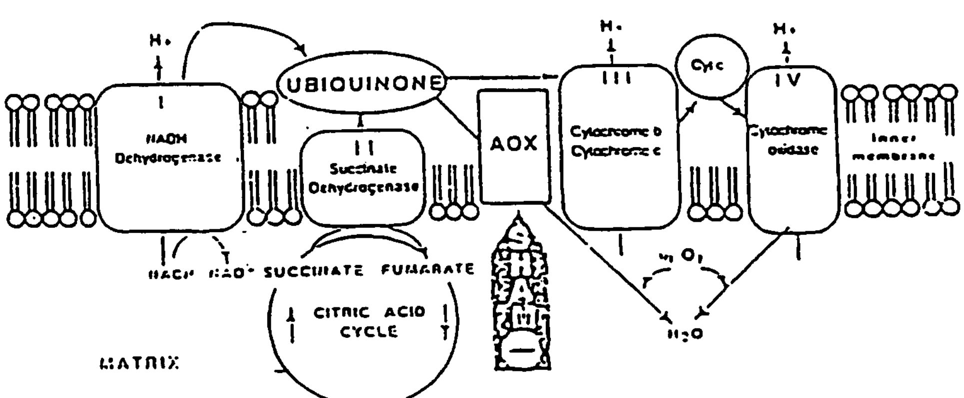


Fig. 3A

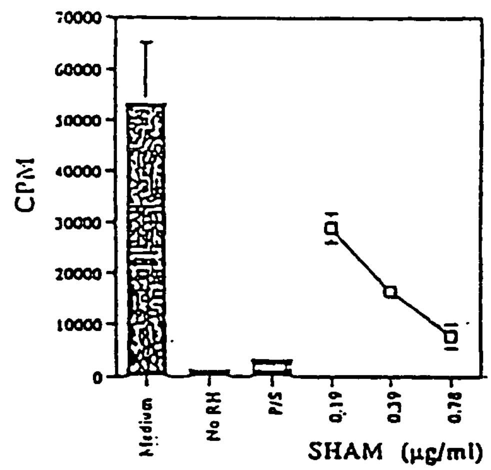

70000
60000
50000
40000
30000
20000
10000
0
CPM
Medium
No RH
P/S
0.19
0.39
0.78
SHAM (μg/ml)

Fig. 3B

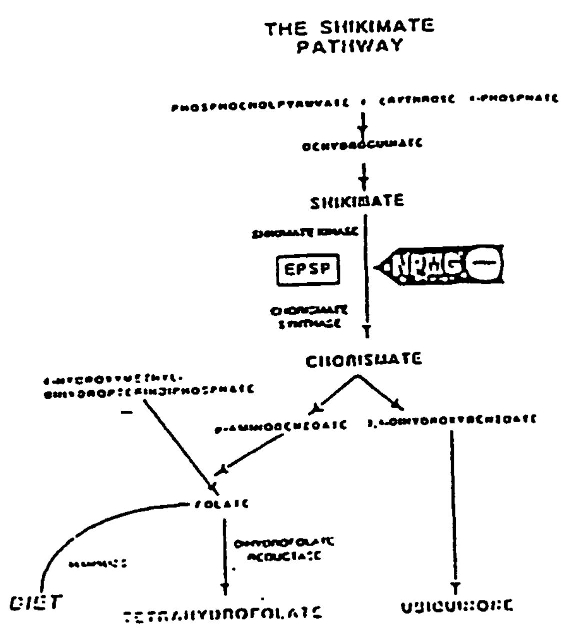

THE SHIKIMATE PATHWAY
SHIKIMATE
EPSP
CHORISMATE
DIET

Fig. 4A

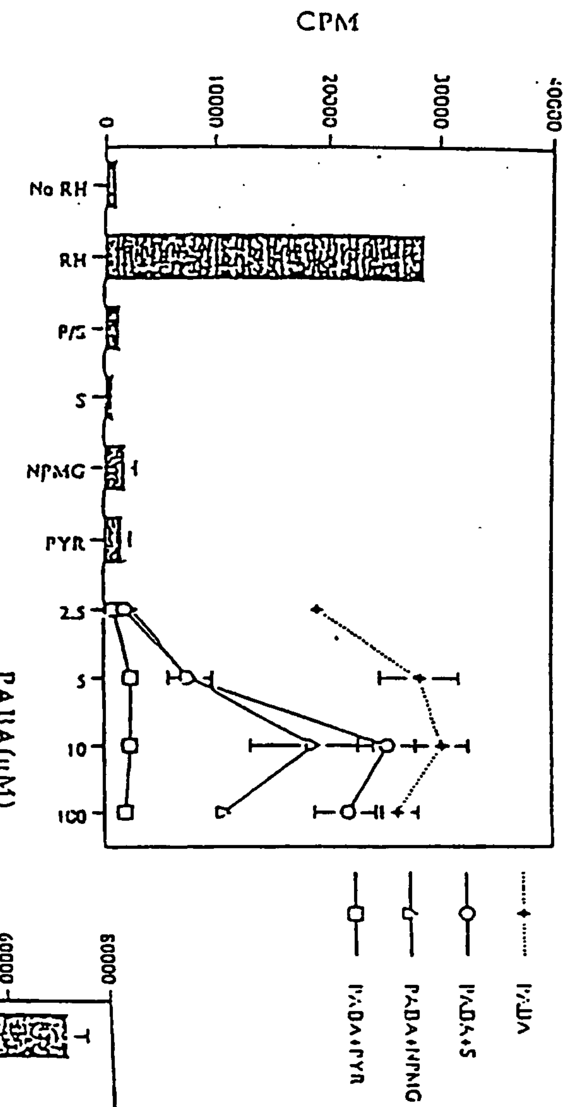

CPM
0
10000
20000
30000
40000
No RH
RH
P/S
S
NPMG
PYR
2.5
5
10
100
PABA(μM)
PABA+PYR
PABA+NPMG
PABA+S
PABA

Fig. 4C

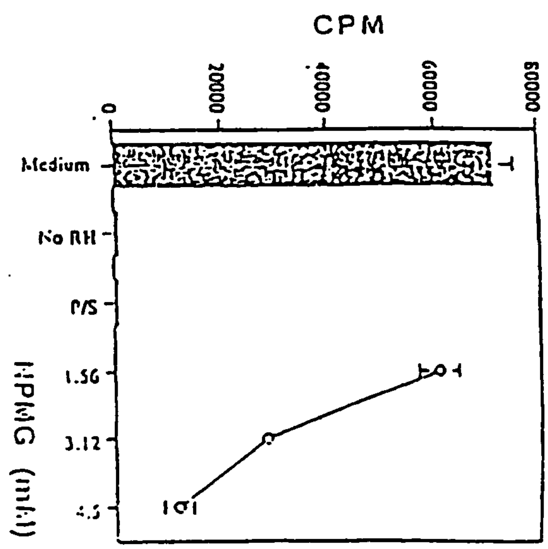

CPM
0
20000
40000
60000
80000
Medium
No RH
P/S
1.56
3.12
6.5
NPMG (mM)

Fig. 4B

Fig. 4D

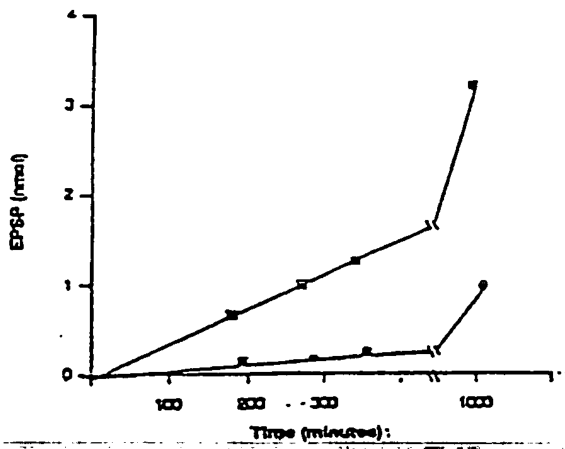


Fig. 4E

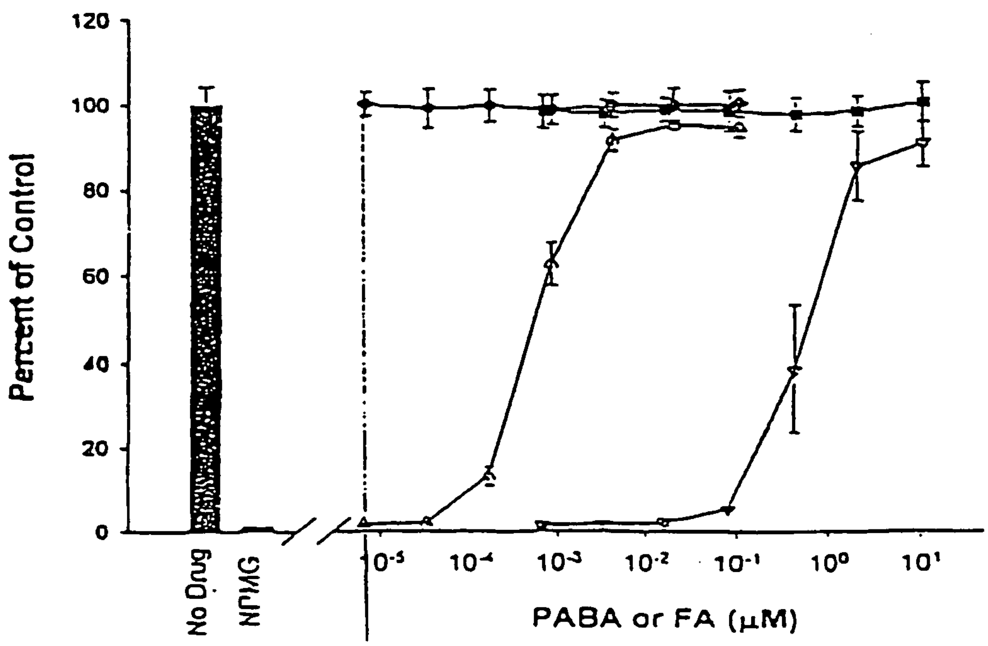


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Fig: 5

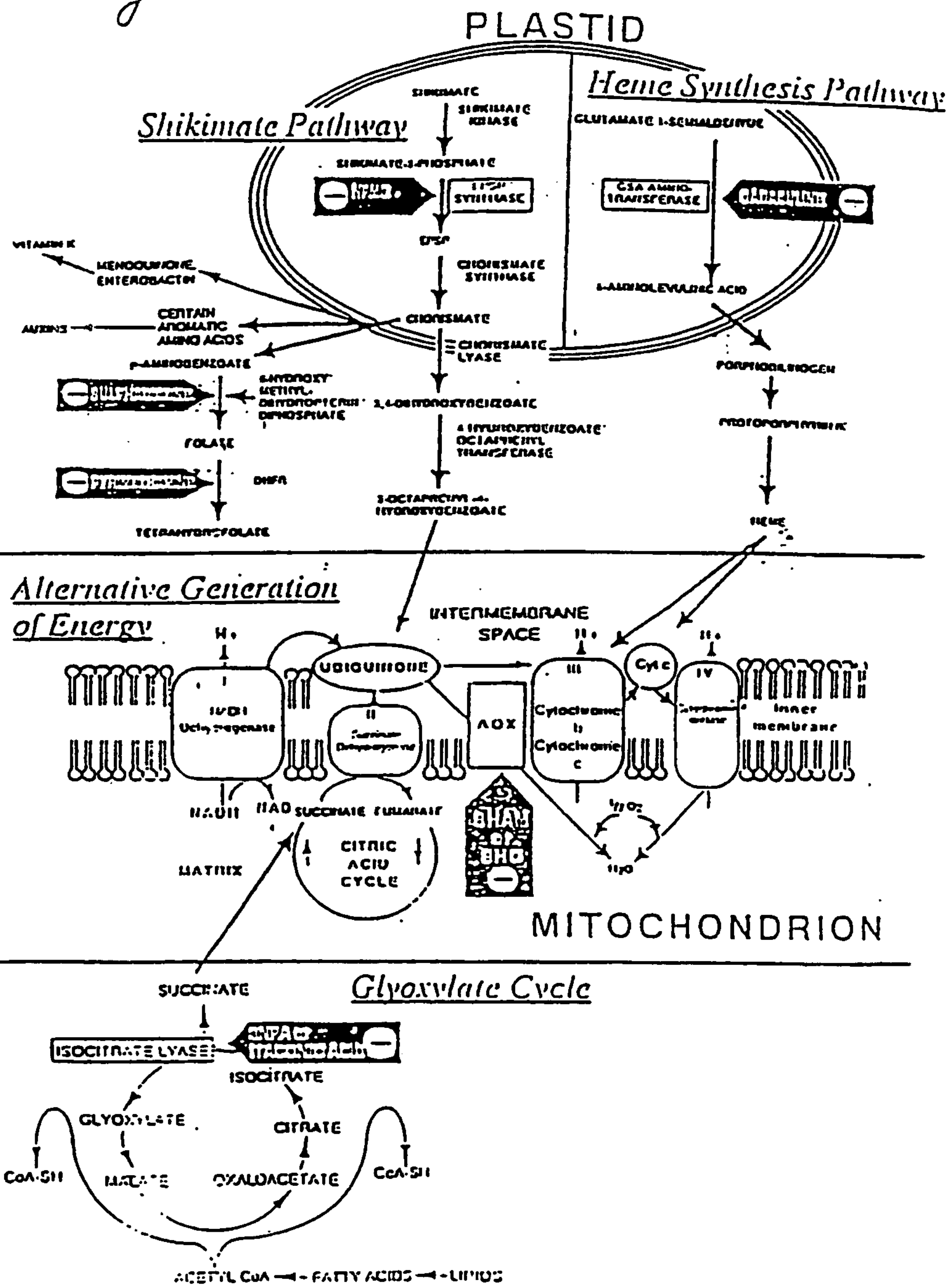
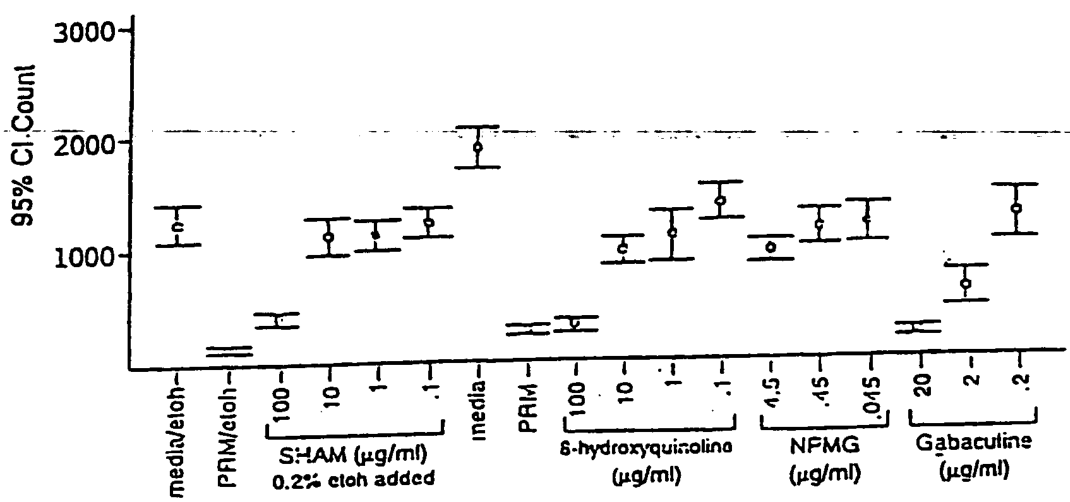

PLASTID
Shikimate Pathway
Heme Synthesis Pathway
CHORISMATE
CERTAIN AROMATIC AMINO ACIDS
FOLATE
DHFR
CHORISMATE LYASE
GSA AMINO-TRANSFERASE
PORPHOBILINOGEN
HEME
Alternative Generation of Energy
INTERMEMBRANE SPACE
UBIQUINONE
AOX
Cyt c
IV
Inner membrane
NADH
NAD
SUCCINATE FUMARATE
CITRIC ACID CYCLE
MATRIX
MITOCHONDRION
SUCCINATE
Glyoxylate Cycle
ISOCITRATE LYASE
ISOCITRATE
GLYOXYLATE
CITRATE
OXALOACETATE
CoA-SH
CoA-SH

Fig: 6


3000
2000
1000
95% CI.Count
media/etoh
PRM/etoh
100
10
1
.1
SHAM (µg/ml)
0.2% etoh added
media
PRM
100
10
1
.1
8-hydroxyquinoline
(µg/ml)
4.5
.45
.045
NFMG
(µg/ml)
20
2
.2
Gabaculine
(µg/ml)

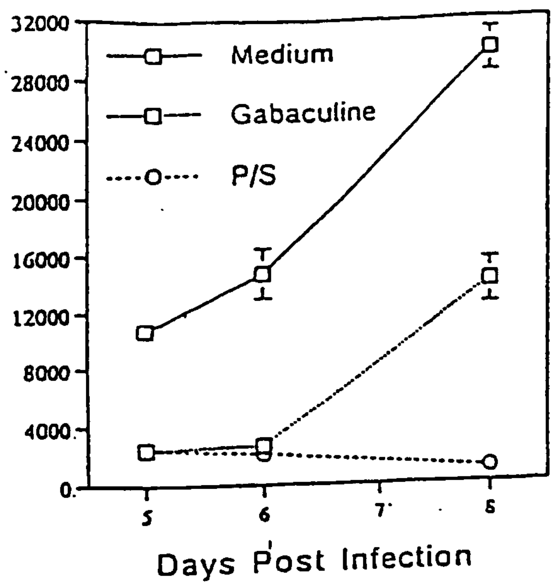

32000
28000
24000
20000
16000
12000
8000
4000
0
Medium
Gabaculine
P/S
5
6
7
8
Days Post Infection

Fig. 7

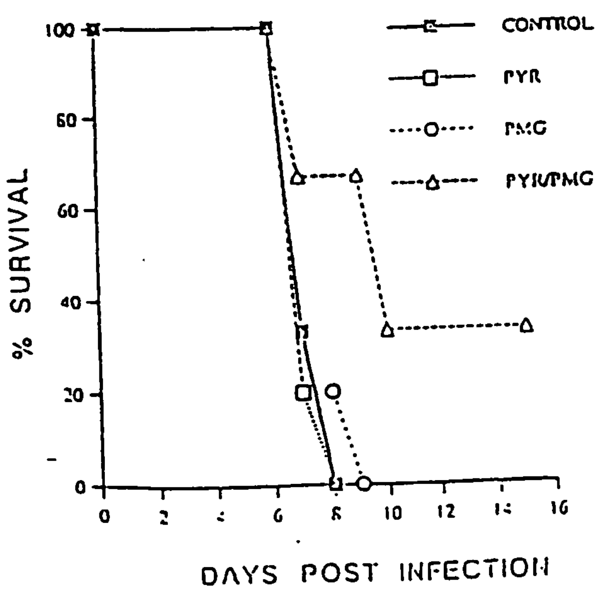

100
80
60
40
20
0
% SURVIVAL
CONTROL
PYR
PMG
PYR/PMG
0
2
4
6
8
10
12
14
16
DAYS POST INFECTION

Fig. 8

Fig. 9(1)

CT CAT CTT CTC GGT TTC 17

ACT TTT CTT TGA GTG CCT GTG TGA GAG ACG GTC GTC GCA ACA AGA ATC 65

TCC TCC GCT CAC GCC TTT CCT CAC AGT CCT GTT TTT CCT CCA GCT GTC 113

ACA CAT CCC GCT CGT TCC GCT GCA TCT CCT CAC ATT TCT TGC AGT CAG 161

ATG TCT TCC TAT GGA GCC GCT CTG CGC ATA CAC ACT TTC GGT GAA TCT 209

M S S Y G A A L R I H T F G E S 16

CAC GGC TCA GCC GTT GGG TGT ATA ATC GAC GGG CTG CCT CCT CGC CTC 257

H G S A V G C I I D G L P P R L 32

CCT CTT TCT GTC GAA GAT GTT CAG CCT CAA TTA AAT CGC AGA AGA CCC 305

P L S V E D V Q P Q L N R R R P 48

GGC CAA GGG CCT CTC TCG ACG CAG CGG AGA GAG AAA GAT CGA GTC AAC 353

G Q G P L S T Q R R E K D R V N 64

ATA CTC TCC GGT GTT GAA GAC GGA TAT ACA CTC GGT ACT CCC CTG GCG 401

I L S G V E D G Y T L G T P L A 80

ATG CTC GTC TGG AAT GAA GAC CGG CGG CCC CAG GAC TAC CAC GCC CTC 449

M L V W N E D R R P Q D Y H A L 96

GCG ACA GTC CCG CGT CCA GGT CAC GGG GAT TTC ACC TAC CAT GCA AAG 497

A T V P R P G H G D F T Y H A K 112

TAC CAC ATT CAC GCG AAA AGC GGG GGC GGT CGG AGC AGC GCG CGG GAG 545

Y H I H A K S G G G R S S A R E 128

ACT TTG GCG CGC GTC GCC GCT GGA GCA GTC GTT GAG AAG TGG CTA GGC 593

T L A R V A A G A V V E K W L G 144

ATG CAC TAC GGC ACC AGC TTC ACA GCT TGG GTC TGT CAG GTT GGT GAT 641

M H Y G T S F T A W V C Q V G D 160

GTC TCT GTG CCC CGA TCG CTC CGA AGA AAG TGG GAG CGG CAG CCG CCA 689

V S V P R S L R R K W E R Q P P 176

ACT CGC CAA GAC GTC GAT CGC CTT GGC GTG GTC CGC GTG AGC CCA GAT 737

T R Q D V D R L G V V R V S P D 192

GGA ACC ACA TTT CTC GAC GCG AAC AAC CGC CTT TAC GAC GAG CGA GGA 785

G T T F L D A N N R L Y D E R G 208

GAG GAA CTC GTC GAG GAG GAA GAC AAA GCC AGG CGT CGG CTT CTT TTC 833

E E L V E E E D K A R R R L L F 224

GGA GTC GAC AAC CCG ACG CCA GGA GAA ACA GTG ATT GAG ACC AGG TGC 881

G V D N P T P G E T V I E T R C 240

CCG TGC CCC TCC ACA GCT GTT CGC ATG GCT GTG AAA ATC AAC CAG ACC 929

P C P S T A V R M A V K I N Q T 256

CGA TCT CTG GGC GAT TCG ATT GGC GGA TGC ATC TCC GGT GCA ATC GTG 977

R S L G D S I G G C I S G A I V 272

CGG CCA CCG CTG GGC CTC GGC GAG CCG TGT TTC GAC AAA GTG GAG GCG 1025

R P P L G L G E P C F D K V E A 288

GAG CTG GCG AAG GCG ATG ATG TCG CTC CCT GCT ACG AAA GGG TTT GAG 1073

E L A K A M M S L P A T K G F E 304

ATT GGC CAG GGC TTT GCG AGT GTC ACG TTG CGA GGC AGC GAG CAC AAC 1121

I G Q G F A S V T L R G S E H N 320

GAC CGC TTC ATT CCC TTC GAG AGA GCG TCC TGT TCA TTC TCG GAA TCA 1169

D R F I P F E R A S C S F S E S 336

GCC GGG ATC ACG ATC AAG CAT GAA AGA GAT GGG TGT TCA GGT GCT ACA 1217

TO FIG. 9(1)

A A S T I K H E R D G C S A A T 352

CTC TCA CGG GAG CGA GCG AGT GAC GGT AGA ACA ACT TCT CGA CAT GAA 1265

L S R E R A S D G R T T S R H E 368

GAG GAG CTG GAA AGG CGG CGG GAG CGC ATA CAG CGC GAT ACC CTC CAT 1313

E E V E R G R E R I Q R D T L H 384

GTT ACT GGT GTA GAT CAG CAA AAC GGC AAC TCC GAA GAT TCA GTT CGA 1361

V T G V D Q Q N G N S E D S V R 396

TAC ACT TCC AAA TCA GAG GCG TCC ATC ACA AGG CTG TCG GGA AAT GCT 1409

Y T S K S E A S I T R L S G N A 416

GCC TCT GGA GGT GCT CCA GTC TGC CGC ATT CCA CTA GGC GAG GGA GTA 1457

A S G G A P V C R I P L G E G V 432

CGG ATC AGG TGT GGA AGC AAC AAC GCT GGT GGA ACG CTC GCA GGC ATT 1505

R I R C G S N N A G G T L A G I 448

ACA TCA GGA GAG AAC ATT TTT TTT CGG GTG GCC TTC AAG CCT GTT TCT 1553

T S G E N I F F R V A F K P V S 464

TCC ATC GGC TTG GAA CAA GAA ACT GCA GAC TTT GCT GGT GAA ATG AAC 1601

S I G L E Q E T A D F A G E M N 480

CAG CTA GCT GTG AAA GGC CGC CAC GAT CCC TGC GTC CTT CCG CGA GCC 1649

Q L A V K G R H D P C V L P R A 496

CCT CCT CTG GTT GAG AGC ATG GCT GCC CTT GTG ATT GGC GAT CTG TGC 1697

P P L V E S M A A L V I G D L C 512

CTC CGC CAG CGC GCC CGG GAA GGG CCG CAC CCC CTT CTC GTC CTT CCT 1745

L R Q R A R E G P H P L L V L P 528

CAA CAC AGT GGT TGC CCA TCT TGC TGA GCT CTA CCT TGT TCC AAA AAC 1793

Q H S G C P S C • 536

TTG TGC ATA CGG GGT ACA CCA GGT TCC TCA CAA GGA GAA TCG TGA GGC 1841

GGT GAC TGG CCA GCG CCA CAG ATT GCT GTT CAT GCA CAA GAA AGA AAA 1889

CAG CGC ATT TCC GCC ACA ACC CAG CTG CAT GAA GTT GCT GGA TAT CGT 1937

TCC GGC GGT GCT CGG CCT TCT TCT CTA CGC TCG CGA TGA TAC GTC GCG 1985

AGC TTC ATC AAG CTC CTT TTG CAT TGT TAG TGG CTC CCA ACA GAA CCC 2033

TTT GTG GAA GGG AAT CTG GTC TCA CGC TTG CAG GAG ACA GTT CGC CTT 2081

TGT TCA CGA AAT AAC GAA GCC AAG CAG CTC AGT TGC ATT CAG CCT GCA 2129

CAC AGT TGC ATT CAG CCT GCA CAC TAA ACA CGG GCG AAA TCG TCG CGT 2177

GAT ATG TAG TTC TTC GGT TGT CAC GGT GAT TGT CGT CGT GTT TGA ACA 2225

ACT AAA CCT TTC TAA TGC TCG ATC TTA AAA AAA AAA AAA AAA AAA AAA 2273

AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA 2312

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Fig. 10(1)

```
T.gondii         -------------------------------------------------MSSYGAALRIH  11
Synechocystis    ------------------------------------------------MGNTFGSLFRIT  12
S.lycopersicum   MASSHLTKQFLGAPFSSFGSGQQPSKLCSSNLRFPTHRSQPKRLEIQAAGNTFGNYFRVT  60
N.crassa         -------------------------------------------------MSTFGHYFRVT  11
H.influenza      -----------------------------------------------MAGNTIGQLFRVT  13
S.cerevisae      -------------------------------------------------MSTFGKLFRVT  11

T.gondii         TFGESHGSAVGCIIDGLPPRLPLSVEDVQPQLNRRRPGQGPLSTQRREKDRVNILSGVED  71
Synechocystis    TFGESHGGGVGVIIDGCPPRLEISPEEIQVDLDRRRPGQSKITTPRKEADQCEILSGVFE  72
S.lycopersicum   TFGESHGGGVGCIIDGCPPRLPLSESDMQVELDRRRPGQSRITTPRKETDTCKISSGTAD 120
N.crassa         TYGESHCKSVGCIVDGVPPGMELTEDDIQPQMTRRRPGQSAITTPRDEKDRVIIQSGTEF  71
H.influenza      TFGESHGIALGCIVDGVPPNLELSEKDIQPDLDRRKPGTSRYTTPRREDDEVQILSGVFE  73
S.cerevisae      TYGESHCKSVGCIVDGVPPGMSLTEADIQPQLTRRRPGQSKLSTPRDEKDRVEIQSGTEF  71

T.gondii         GYTLGTPLAMLVHNEDRRPQDYH--ALATVPRPGHGDFTYHAKYHIHAKSGGGRSSARET 129
Synechocystis    GKTLGTPIAILVRNKDARSQDYN--EMAVKYRPSHADATYEAKYGIRNHQGGGRSSARET 130
S.lycopersicum   GLTTGSPIKVEVPNTDQRGHDYS--EMSLAYRPSHADATYDFKYGVRSVQGGGRSSARET 178
N.crassa         GVTLGTPIGMLVHNEDQPPKDYGNKTHDIYPRPSHADHTYLEKYGVKASSGGGRSSARET 131
H.influenza      GKTTGTSIGMIIKNGDQRSQDYG--DIKDRFRPGHADFTYQQKYGIRDYRGGGRSSARET 131
S.cerevisae      GKTLGTPIAMMIKNEDQRPHDYS--DMDKFPRPSHADFTYSEKYGIKASSGGGRASARET 129

T.gondii         LARVAAGAVVEKHLGMHYGTSFTAHVCQVGDVSVPRSLRRKHERQPPTRQDVDRLGVVRV 189
Synechocystis    IGRVAAGAIAKKILAQFNGVEIVAYVKSIQDIEA-------------------------- 164
S.lycopersicum   IGRVAAGAVAKKILKLYSGTEILAYVSQVHNVVLP------------------------- 213
N.crassa         IGRVAAGAIAEKYLKPRYGVEIVAFVSSVGSEHLFPPTAEHFSFST-------------- 177
H.influenza      AHRVAAGAIAKKYLREHFGIEVRGFLSQIGHIKIAP------------------------ 167
S.cerevisae      IGRVASGAIAEKFLAQNSNVEIVAFVTQIGEIKHHR------------------------ 165

T.gondii         SPGGTTFLDAHHRLYDERGEELVEEEDKARRRLLFGVDHPTPGETVIETRCPCPSTAVRM 249
Synechocystis    -------TVDSNTVTLEQVESH-------------------------IVRCPDEECAEFH 192
S.lycopersicum   -----EDLVDNQIVTLEQIESN-------------------------IVRCPNPEYAEKK 243
N.crassa         ---NPEFLKLVNSITRETVDSFL------------------------PVRCPDAEANKRH 210
H.influenza      ---------QKVGQIDWEKVNSH------------------------PFFCPDESAVEKF 194
S.cerevisae      DSFDPEFQHLLHTITREKVDSHG------------------------PIRCFDASVAGLH 201

T.gondii         AVKIHQTRSLGDSIGGCISGAIVRPFLGLGEPCFDKVEAELAKAMMSLPATKGFEIGQGF 309
Synechocystis    IEKIDQVLRQKDSIGGVVECAIRNAPKGLGEPVFDKLEADLAKAMMSLPATKGFEFGSGF 252
S.lycopersicum   IGAIDYVRVRGDSVGGVVTCIVRNVPRGLGTPVFDKLEAELAKACMSLPATKGFEFGSGF 303
N.crassa         EDLITKFRDHHDSIGGTVTCVIRNVPSGLGEPAFDKLEAMLAHAMLSIPATKGFEVGSGF 270
H.influenza      GELIRELKKEGDSIGAKLTVIAENVPVGLGEPVFDRLDADLAHALMGINAVKGVEIGDGF 254
S.cerevisae      VKEIEKYRGNKDSIGGVVTCVVRNLPTGLGEPCFDKLEAMLAHAMLSIPASKGFEIGSGF 261
```

—— TO FIG. 10(2) ——

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TO FIG. 10(1)

```
T.gondii.          ASVTLRGSEHNDRFIPFERASCSFSESAASTIKHERDGCSAATLSRERASDGRTTSRHEE 369
Synechocystis      AGTLLTGSQHNDEYYLDEAGEWR------------------------------------- 275
S.lycopersicum     AGTFNTGSEHNDEFFHDEHDQIR------------------------------------- 326
N.crassa           GGCEVPGSIHHDPFVSAENTEIPPSVAASGAARNGI------------------------ 306
H.influenza        AVVEQRGSEHRDEHTPHGFESNH------------------------------------- 277
S.cerevisae        QGVSVPGSIHNDPFYFEKETNR-------------------------------------- 283

T.gondii           EVERGRERIQRDTLHVTGVDQQNGHSEDSVRYTSKSEASITRLSGNAASGGAPVCRIPLG 429
Synechocystis      ------------------------------------------------------------
S.lycopersicum     ------------------------------------------------------------
N.crassa           ------------------------------------------------------------
H.influenza        ------------------------------------------------------------
S.cerevisae        ------------------------------------------------------------

T.gondii           EGVRIRCGSHNAGGTLAGITSGEHIFFRVAFKPVSSIGLEQETADFA-GEHHQLAVKGRH 488
Synechocystis      ------TRTHRSGGVQGGISHGEPIIHRIAFKPTATIGQEQKTVSHI-GEETTLAAKGRH 320
S.lycopersicum     ------TKTNRSGGIQGGISHGEIIHMRVAFKPTSTIARKQHTVSRD-KHETELIARGRH 379
N.crassa           PRPKLTTKTHFSGGIQGGISNGAPIYFRVGFKPAATIGQEQTTATYDGTSEGVLAAKGRH 366
H.influenza        -----------AGGILGGISSGQPIIATIALKPTSSITIFGRSINLH-GEAVEVVTKGRH 325
S.cerevisae        ----LRTKTHHSGGVQGGISHGEHIYFSVPFKSVATISQEQKTATYD-GEEGILAAKGRH 338

T.gondii           DPCVLPRAPPLVESHAALVIGDLCLRQRAREGPHPLLVLPQHSGCPSC------------ 536
Synechocystis      DPCVLPRAVPMVEAMAALVLCDHLLRFQAQCKTL-------------------------- 362
S.lycopersicum     DPCVVPRAVPMVEAMVALVLVDQLMTQYAQCHLFPVHLTLQEPLQPSTTKSA-------- 431
N.crassa           DPSVVPRAVPIVEAMAALVIHDAVLAHEARVTAKSLLPFLKQTINSGKDTVGHGVSEHVQ 426
H.influenza        DPCVGIRAVPIAEAMVAIVLLDHLLRFKAQCK---------------------------- 357
S.cerevisae        DPAVTPRAIPIVEAMTALVLADALLIQKARDFSRSVVH---------------------- 376

T.gondii           ------
Synechocystis      ------
S.lycopersicum     ------
N.crassa           ESDLAQ   432
H.influenza        ------
S.cerevisae        ------
```

Fig. 10(2)

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```
                                                   Mature protein starts here
                     .  *  .**  ..  * . .* ***  *.* .*...**...  **  ||
Transit Peptide Wx Zea Mays   MAALATSQLVATRAGLGVPDASTFRRGAAQGLRGARASAAADTLSM-RTS-ARAAPRHQQQARRGGRFPSLVVCASAGMNVVFV
(SEQ ID NO: 44)                                                            |
                                                                    Processing site

Homologous Portion of T. gondii AroC          SCSFSESAASTIKHERDGCSAATLSRERASDGRTTSRHEEEVERG
(SEQ ID NO: 43)
```

Fig. 11

Fig. 12 (1)

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```
                                                    CT CGA GTT      6

TTT TTT TTT TTT TTT TTT TTG ATA CAT AAT AAT CAA GAG TTC TTT ATA    56

CTA ACA GAC TTA TTT AAT GTA TTA TTT TTG GTA AAC AAA AAA AAC ATT   104

ATG AGC ACA TAT GGG ACT TTA TTA AAA GTA ACA TCC TAC GGA GAA AGT   152
 M   S   T   Y   G   T   L   L   K   V   T   S   Y   G   E   S     16
CAT GGG AAA GCT ATT GGG TGT GTG ATC GAT GGG TTT TTA TCC AAT ATA   200
 H   G   K   A   I   G   C   V   I   D   G   F   L   S   N   I     32
GAA ATA AAT TTT GAT TTA ATA CAA AAA CAA TTA GAT AGA CGA AGA CCA   248
 E   I   N   F   D   L   I   Q   K   Q   L   D   R   R   R   P     48
AAT CAA TCA AAA CTA ACT AGT AAT AGA AAC GAA AAA GAT AAA CTT GTT   296
 N   Q   S   K   L   T   S   N   R   N   E   K   D   K   L   V     64
ATA CTT TCA GGA TTT GAT GAA AAT AAA ACA TTA GGT ACA CCT ATT ACA   344
 I   L   S   G   F   D   E   N   K   T   L   G   T   P   I   T     80
TTT TTA ATA TAT AAT GAA GAT ATT AAA AAA GAA GAT TAT AAT TCT TTT   392
 F   L   I   Y   N   E   D   I   K   K   E   D   Y   N   S   F     96
ATA AAT ATT CCT AGA CCA GGA CAT GGA GAT TAT ACC TAT TTT ATG AAA   440
 I   N   I   P   R   P   G   H   G   D   Y   T   Y   F   M   K    112
TAT CAT GTT AAA AAT AAA AGT GGA AGT AGT AGA TTT TCT GGA AGA GAA   488
 Y   H   V   K   N   K   S   G   S   S   R   F   S   G   R   E    128
ACA GCC ACA AGA GTT GCT GCT GGG GCG TGC ATT GAA CAA TGG CTT TAT   536
 T   A   T   R   V   A   A   G   A   C   I   E   Q   W   L   Y    144
AAA TCT TAT AAT TGT TCT ATT GTT AGT TAT GTA CAT TCA GTT GGG AAT   584
 K   S   Y   N   C   S   I   V   S   Y   V   H   S   V   G   N    160
ATA AAG ATA CCT GAA CAA GTC AGC AAA GAA TTG GAA AAT AAA AAT CCA   632
 I   K   I   P   E   Q   V   S   K   E   L   E   N   K   N   P    176
CCC TCA AGA GAT TTA GTA GAT TCT TAT GGA ACC GTT AGA TAT AAT GAA   680
 P   S   R   D   L   V   D   S   Y   G   T   V   R   Y   N   E    192
AAA GAA AAA ATA TTT ATG GAT TGT TTT AAT AGA ATA TAT GAT ATG AAT   728
 K   E   K   I   F   M   D   C   F   N   R   I   Y   D   M   N    208
GCT TCT ATG TTA AAA ACT GAT GAA TAT AAT AAA AAC ACA TTG ACT ATT   776
 A   S   M   L   K   T   D   E   Y   N   K   N   T   L   T   I    224
CCT TCA ATA GAT AAC ACG TAT ATA AAT GTA AAA ACT AAT GAA TGT AAT   824
 P   S   I   D   N   T   Y   I   N   V   K   T   N   E   C   N    240
ATA AAT CAG GTT GAT AAT AAT CAT AAC AAT TAT ATT AAT GAT AAG GAT   872
 I   N   Q   V   D   N   N   H   N   N   Y   I   N   D   K   D    256
AAC ACT TTT AAT AAT TCT GAA AAA TCG GAT GAA TGG ATT TAT TTA CAA   920
 N   T   F   N   N   S   E   K   S   D   E   W   I   Y   L   Q    272
ACA AGA TGT CCA CAT CCA TAT ACT GCT GTA CAA ATT TGT TCT TAT ATT   968
 T   R   C   P   H   P   Y   T   A   V   Q   I   C   S   Y   I    288
TTG AAA CTA AAA AAT AAA GGA GAT AGT GTT GGG GGT ATT GCT ACA TGC  1016
 L   K   L   K   N   K   G   D   S   V   G   G   I   A   T   C    304
ATT ATA CAA AAT CCT CCT ATA GGT ATT GGA GAA CCT ATT TTT GAC AAA  1064
 I   I   Q   N   P   P   I   G   I   G   E   P   I   F   D   K    320
TTG GAA GCT GAG CTA GCC AAA ATG ATT TTA TCT ATT CCA CCC GTG AAA  1112
 L   E   A   E   L   A   K   M   I   L   S   I   P   P   V   K    336
```

TO FIG. 12 (2)

TO FIG. 12(1)

McLeod *et al.*

```
GGA ATA GAA TTC GGG AGT GGA TTT AAT GGT ACA TAT ATG TTT GGC TCA   1160
 G   I   E   F   G   S   G   F   N   G   T   Y   M   F   G   S    352
ATG CAT AAT GAT ATC TTC ATA CCT GTA GAA AAT ATG TCT ACA AAA AAA   1208
 M   H   N   D   I   F   I   P   V   E   N   M   S   T   K   K    368
GAA AGT GAT TTA TTA TAT GAT GAT AAA GGT GAA TGT AAA AAT ATG TCT   1256
 E   S   D   L   L   Y   D   D   K   G   E   C   K   N   M   S    384
TAT CAT TCA ACG ATT CAA AAT AAT GAG GAT CAA ATA TTA AAT TCA ACT   1304
 Y   H   S   T   I   Q   N   N   E   D   Q   I   L   N   S   T    400
AAA GGA TTT ATG CCT CCT AAA AAT GAC AAG AAT TTT AAT AAT ATT GAT   1352
 K   G   F   M   P   P   K   N   D   K   N   F   N   N   I   D    416
GAT TAC AAT GTT ACG TTT AAT AAT AAT GAA GAA AAA TTA TTA ATT ACA   1400
 D   Y   N   V   T   F   N   N   N   E   E   K   L   L   I   T    432
AAA ACA AAT AAT TGT GGT GGG ATT TTA GCT GGC ATT TCA ACA GGA AAC   1448
 K   T   N   N   C   G   G   I   L   A   G   I   S   T   G   N    448
AAT ATT GTT TTT AGA TCA GCA ATC AAA CCT GTA TCA TCA ATA CAA ATA   1496
 N   I   V   F   R   S   A   I   K   P   V   S   S   I   Q   I    464
GAA AAA GAA ACA AGT GAT TTT TAT GGA AAT ATG TGT AAC TTG AAA GTT   1544
 E   K   E   T   S   D   F   Y   G   N   M   C   N   L   K   V    480
CAA GGG AGA CAT GAT AGC TGT ATT TTA CCA AGA TTA CCA CCC ATT ATT   1592
 Q   G   R   H   D   S   C   I   L   P   R   L   P   P   I   I    496
GAA GCA TCT TCT TCA ATG GTT ATA GGA GAT TTA ATA TTA CGA CAA ATA   1640
 E   A   S   S   S   M   V   I   G   D   L   I   L   R   Q   I    512
TCA AAG TAT GGA GAT AAA AAG TTG CCA ACA TTG TTT AGG AAT ATG TAA   1688
 S   K   Y   G   D   K   K   L   P   T   L   F   R   N   M   ·    527
CAT AAT GAT TTT GTA ATC CTC AAT TAA AAT GAA AAA TTA TAA AAT ATA   1736

TAT TTT ATA TAT ATA TAT AAA ATA TAT ATA TAT ATA TAT AAA ATA TAA   1784

ATA TAT GTA TAA TAA TTC AAT TTG CGC AAT CGA TCA AAA TAC ATT TCG   1832

TCT AC                                                            1837
```

Fig. 12(2)

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Genomic Sequence Chorismate Synthase

Figure13.

```
   1 GAATTCTGCA GTTCTTTTGA ATATATGGCT GCCCACTACC CGTAGGTATT TGCGACGCAG
  61 CGCTTGCGTC ACTCGGTGGC GTGACACACA ACCTGCACTG GCCGCCACTC GCGCGCATCC
 121 ACGGTAGAGC TAACGAGTCT GCGATGGGGT TAGAGACGCA CACCTTTGAC TCCCGGGGCC
 181 TACGGAGACG ACGCGGACGC GTGTCTCCCC TTTTCGCTCT TTTTACTGTA CGCTGGTAAA
 241 ACGACTTTTC GACGCAGCAT GGTTCTCATC TTCTCGGTTT CACTTTTCTT TGAGTGCCTG
 301 TGTGAGAGAC GGTCGTTGCA ACAAGAATCT CCTCCGCTCA CGCCTTTCCT CACAGTCCTG
 361 TTTTTCCTCC AGCTGTTACA CATCCCGCTC GTTCCGCTGC ATCTCCTCAC ATTTCTTGCA
 421 GTCAGATGTC TTCCTATGGA GCCGCTCTGC GCATACACAC TTTCGGTGAA TCTCACGGCT
 481 CAGCCGTTGG GTGTATAATC GACGGGCTGC CTCCTCGCCT CCCTCTTTCT GTCGAAGATG
 541 TTCAGCCTCA ATTAAATCGC AGAAGACCCG GCCAAGGGCC TCTCTCGACG CAGCGGAGAG
 601 AGAAAGATCG AGTCAATATA CTCTCCGGTG TTGAAGACGG ATATACACTC GGTGAGGGAA
 661 GAAACTACAG ACGTCATGTG CCTGTGCCAG CACATAACTG CAGATTCATA TATATATATA
 721 CATATACAGA TGTGTATTTT GTGTGTATAG TTAAGCAGAG GATGGTATTG AAAATGGCTG
 781 TCGGTGTATT TTTATTTGCC CTGTGGCGCT TTTGGAGAAG GCCCTGGGGA AACGGAAGCC
 841 CTGGCACAAG GGCTGCTGGC TAAGCTTCAG AAACCGCAGT TAATAGCTCG AAAGTACCGT
 901 ATCCAAACGT TCTCTTTTAT CCACACAGTG TGTTGGACAC AAGCGAAGCC GAAAAGTGTC
 961 TTGCACGTGG TGAGTTTTCG GTGACAAAAC ACACGCGCCA CTCCGTAGAA ATACCGGATC
1021 CGAGTTTACC TGCTGCAGGC TTCGGAACGC TGCTTTGTTC CGAAGATGGC CTCGTGGTTT
1081 CGATGGGAAA TTGGAGGGTG CAAAAGTGCC CGGCGCTCGT GGCCTGCGCC ATCTGGCATC
1141 GTGGACTGGC TGTCTATTGT GATCCTCGCG TCCCTTCCAA AAAATCATTT TTTTCTGCTT
1201 CGCCTTCTCG TTCGTGTTAC CGGGATCCGT CTGCAGGTAC TCCCCTGGCG ATGCTCGTCT
1261 GGAATGAAGA TTGGCGGTCC CAGGACTACC ACGCCCTCGC GACAGTCCCG CGTCCAGGTC
1321 ACGGGGATTT TACCTATTAT GCAAAGTACC ACATTCACGC GAAAAGCGGG GGCGGTCGGA
1381 GCAGCGCGCG GGAGATTTTG GCGCGCGTCG CCGCTGGAGC AGTCGTTGAG AAGTGGCTAG
1441 GCATGCACTA TGGCACTAGC TTCACAGCTT GGGTCTGTCA GGTGAGACGA AGCCCAGAAG
1501 GTTACAGGAG AGTGGATGAA AAGACAGAGA TAGACAGGTC TTCGCTGGAG GCAGTACGCG
1561 GATGGAAGAC AACGTTTAGG CGCTTTCCGA TTCATGGGGC AAGCGTGGCT AATTTTCCAT
1621 GACTCGACAG TGGTGATTCT AGGATCGCGT CGGTTTTTGA TGCCTGGTTC TCTCACGCCT
1681 TAGGTTGGTG ATGTCTTTGT GCCCCGATCG CTCCGAAGAA AGTGGGAGCG GCAGCCGCCA
1741 ACTCGCCAAG ACGTCGATCG CCTTGGCGTG GTCCGCGTGA GCCCAGATGG AACCACATTT
1801 CTCGACGCGA ACAACTTGCT TTACGACGAG CGAGGAGAGG AACTCGTCGA GGAGGAAGAC
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1921 GAGACCAGGT GCCCGTGTCC CTCCACAGCT GTTCGCATGG CTGTGAAAAT CAACCAGGTG
1981 AGGTGGAGCA GTGCGATGAG CCATCTGTTC ACTGGATCCG TAAACGCGAA GGTCATCCGT
2041 GGGGGAAAAA AGTGAATTTA CGGAAGGTGA GCTGGCTTTG GCCGTGACAC GTCTAGTCTA
2101 CCCTGCAGAC TTACCATTTG GCGAATAGCA AAGCAGCGGG GGAAGGCGTC ACCCGGAGAA
2161 GGGTGTCGAG TAGTGCGTCC ACCCAGAGGC TCGGAAGACC TCCGCGAACG TTGATGGTGT
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2401 CCTCGGTAAG TAGTCTTGTT TTCTGTGTTT CCTCGGCTCC TATACAGCAC CTGACCACGT
2461 TTCTAGGTGG TGTGGCGACA GGTCGGACCT ATATTCGAGA CGTGCACAGT TCGTCCAAAT
2521 TGCTCGTTCC ATGCACTAGC ATCTCCTTGC CAGACACCCC ACACACCGCA TAGGTTTGCT
2581 TGACAAATGA AACTGATAAA TACGACCTGC GGGGACTTGT GACAACGTTG CCCTTTTGCC
2641 GTTTTCCTGC GAGGTCGTGA CTGAGGCGCT GGTGAAGAGC GAGACTGGGC CGAGGCGTGT
2701 GTTTCCATGC AAACAGAAAG CAGGCTGATA GAGACATGCA AACGAGCGGA CGTGGAAGCG
2761 CAGTGCTGAA TGCATGAACT AACTAAAGGT GCACACACCT GCGCACCACC CGAGATGCAG
2821 CGACCGACGG GACATTTTTG TGAGGTGCAG ATGACTCTGC ATCAAGAATC AGTGCCTCAG
2881 AGACCCTTTT TTTTGTGTAG TTTCTCAGTG CGGCAGAAAG AGTTTTCGTT GCTCTGTTCA
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3001 TCAGAGAGTG TAAGGGAGAG TTTTTTGAAT TATGTTTTCT CTGATTTTTT GCTGGAGGTC
3061 TGTGCATGTA GGTGAGTTGT GTTTCGACAA AGTGGAGGCG GAGCTGGCGA AGGCGATGAT
3121 GTCGCTCCCT TTTACGAAAG GGTTTGAGGT ATGTGTGCAA CTTTGTCCAG AGAGGTGATA
3181 ATTGAGCACG ACGCATGTAA TTTGTGGTCA GGCCCAATAT GTACAGCTTA GTTTCCACCG
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Figure13. *continued*

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3241 AAGAAATCAA CACTGGTCGG GTCTTTTCAC GCCACCTGTG GCCTGTCGCT TTCACTCTTT
3301 GCCTGGGATA GATGTGAGGC ACACTTCGTC AACACCTTGC CGCTGGCTCT ATATCGGACG
3361 CCACCCTGAA TCGCGTTGCG AATGTTTTCT TTTGCATTCG TGATGCATCC GTCTGTGTTG
3421 ACAGATTGGT CAGGGTTTTG CGAGTGTCAC GTTGCGAGGC AGCGAGCACA ACGACCGCTT
3481 CATTCCCTTC GAGAGAGCGT CGTGTTCATT CTCGGAATCA GCCGCGAGCA CGATCAAGCA
3541 TGAAAGAGAT GGGTGTTCAG CTGCTACACT CTCACGGGAG CGAGCGAGTG ACGGTAGAAC
3601 AACTTCTCGA CATGAAGAGG AGGTGGAAAG GGGGCGGGAG CGCATACAGC GCGATACCCT
3661 CCATGTTACT GGTGTAGATC AGCAAAACGG CAACTCCGAA GATTCAGTTC GATACACTTC
3721 CAAATCAGAG GCGTCCATCA CAAGGCTGTC GGGAAATGCT GCCTCTGGAG GTGCTCCAGT
3781 CTGCCGCATT CCACTAGGCG AGGGAGTACG GATCAGGTGT GGAAGCAACA ACGCTGGTGG
3841 AACGCTCGCA GGCATTACAT CAGGTGGGTC CCGACCCGTT ACTCGCGCTC CGCTTCCTGT
3901 CCAGTTCCGG CGTTCGACAG CACTCGTTCA AAGTGGTTGG TTTTCTGGCC AGTGGCAGCA
3961 TTGGCTGTAA AGAACATACT GTTGCTGGCT GCTTTCAATA GGTGTAAAAA AAACTGGTGT
4021 CCTTTCATTC AGTCTACAGC TCTGATGCAC CTTTCTGCTG CCCACGTGAG TCCTTGCTGC
4081 GGCCATCGAC TCAGATAGAA CAAGATCCCC CAGATACAAG AGAAATGTCT TGAGCCAAGA
4141 AGACGGCTGT CTAATTACAC GATACGGACA TCAGTAATGA GATTTTAACA GAGGGGCTTC
4201 CAGCATCGCT GCAGGATGTC GCGTCGCGAC CTCAGGTTGT TGATTCTGTG CTGAGAGACA
4261 CACATTGTGC AACTGCTGCC TGCCCTGTCT TGTTCGTGCG TCCGTGGTGA AGTACCATCG
4321 ACGTGATGAA CAGCCTGAAT GCAGACGTCG TCTAACGGGG TGCGCACCAC CCCAAGAGGA
4381 CGGTGTGACT ACGTCGGTGG CGTGGATTGA TGTGTGTTCA TCAGGAGAGA ACATTTTTTT
4441 TCGGGTGGCC TTCAAGCCTG TTTCTTCCAT CGGCTTGGAA CAAGAAACTG CAGACTTTGC
4501 TGGTGAAATG AACCAGCTAG CTGTGAAAGG TAAGAGGCAT TTGCTTATTT GGGTCTCGAC
4561 TTAGGCGGTC ACATTGCCAT TCACTCTTAT CAACATTTGC AAGGTCGAAA TCTGTGGTGC
4621 ACATGGATGC AGTCGAGGGC GGGTCACTCA CATTGCATTT TCTCCACACG CTCGCCCAAC
4681 AAGAAACTGG CTTGGTGTTC TCGTGAATTC GTTGACAGGC CGCCACGATC CCTGCGTCCT
4741 TCCGCGAGCC CCTCCTCTGG TTGAGAGCAT GGCTGCCCTT GTAAGCCGGC AACATAATCT
4801 GGGAAAACGA AAACGATTGC CAGAGCGGGG ATGGGCACAA CACGGATCCG TGATGTTCCG
4861 TAGTACCTCG AGTCTCCCTG AGTCTTGTGC GGGATTGGTG ACTGCACCCA AAATGTGTTG
4921 GAATCGAACG CTGGATCAGT GAACTCCTTG GCTGATGTCT CTCAACCGTA TGACTGCTTC
4981 TCAAACAGCT CATATAACAC CCGTGGGAAC TGTAGCAACA ATTTTCCTTC ACAATTTGGC
5041 CCGGGTCCGT GCAAAGACAT TATGCAAAGC AGCCCTCAGT CGTGTGCCTC GCTTGCGTGC
5101 AGTTTCACGT AAGACTGGCA TGAGGACCGA ACTACCGTGC AGGGAAACAT GCTGACGTCC
5161 CCCGTAGAAT GTTCTTGAGG GAATCTGCGG TGTGGCCTCC TTCCTCGAAC AGTAGGACAA
5221 TCCTGTCTTC TTGTCGCCTG TAGATCCTGG CCGTTCATTA ACCCCTCTTT GAATTCGTCA
5281 CTTGCCTCGA TGACATGCCC CCTTAGGTGA TTGGCGATCT GTGCCTCCGC CAGCGCGCCC
5341 GGGAAGGGCC GCACCCCCTT CTCGTCCTTC CTCAACACAG TGGTTGCCCA TCTTGCTGAG
5401 CTCTACCTTG TTCCAAAAAC TTGTGCATAC GGGGTACACC AGGTTCCTCA CAAGGAGAAT
5461 CGTGAGGCGG TGACTGGCCA GCGCCACAGA TTGCTGTTCA TGCACAAGAA AGAAAACAGC
5521 GCATTTCCGC CACAACCCAG CTGCATGAAG TTGCTGGATA TCGTTCCGGC GGTGCTCGGC
5581 CTTCTTCTCT ACGCTGGCGA TGATACGTCG CGAGCTTCAT CAAGCTCCTT TTGCATTGTT
5641 AGTGGCTCCC AACAGAACCC TTTGTGGAAG GGAATCTGGT CTCACGCTTG CAGGAGAGAG
5701 TTCGCCTTTG TTCACGAAAT AACGAAGCCA AGCAGCTCAG TTGCATTCAG CCTGCACACA
5761 GTTGCATTCA GCCTGCAAAC TAAACACGGG CGAAATCGTC GCGTGATATG TAGTTCTTCG
5821 GTTGTCACGG TGATTGTTGT CGTGTTTGAA CAACTAAACG TTTCTAATGC TGGATCCGAA
5881 TTC
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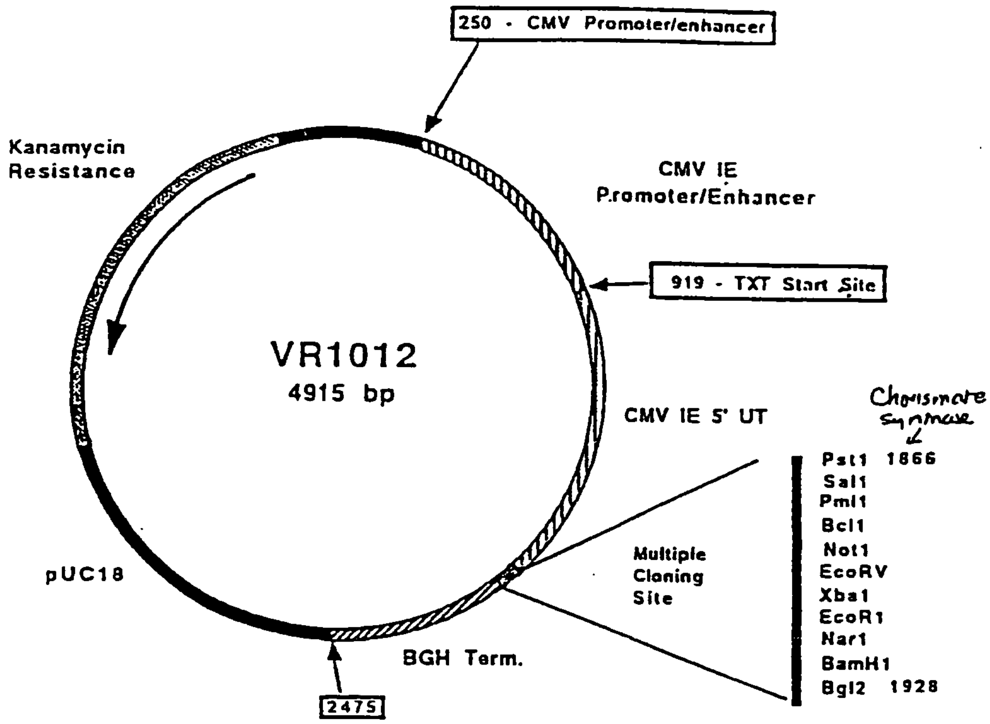
Fig. 14A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Kanamycin Resistance
CMV IE Promoter/Enhancer
919 - TXT Start Site
VR1012
4915 bp
CMV IE 5' UT
Chorismate synthase
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Sal1
Pml1
Bcl1
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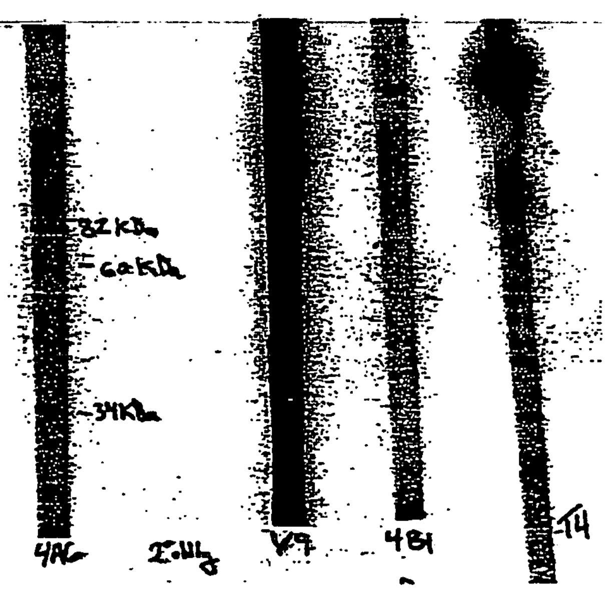
BGH Term.
2475

14B



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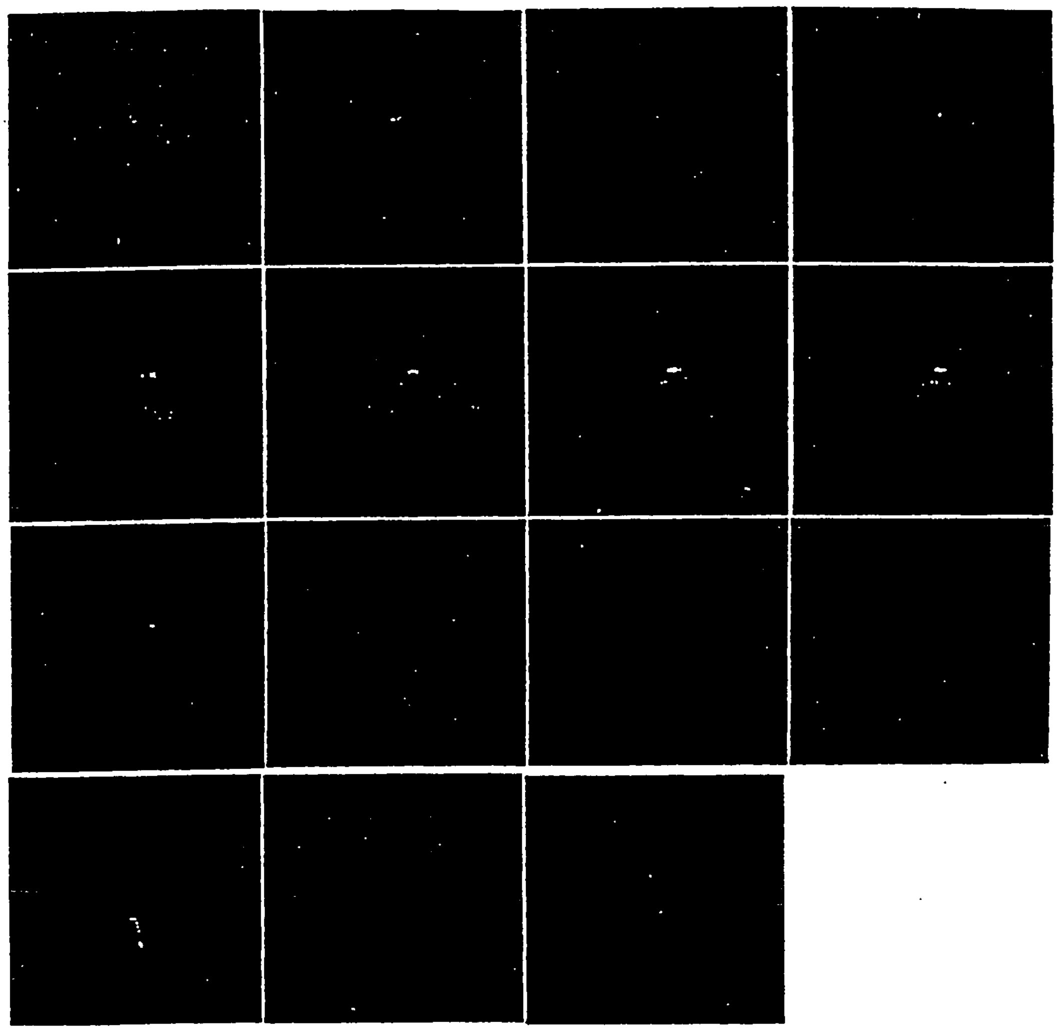

Fig. 15

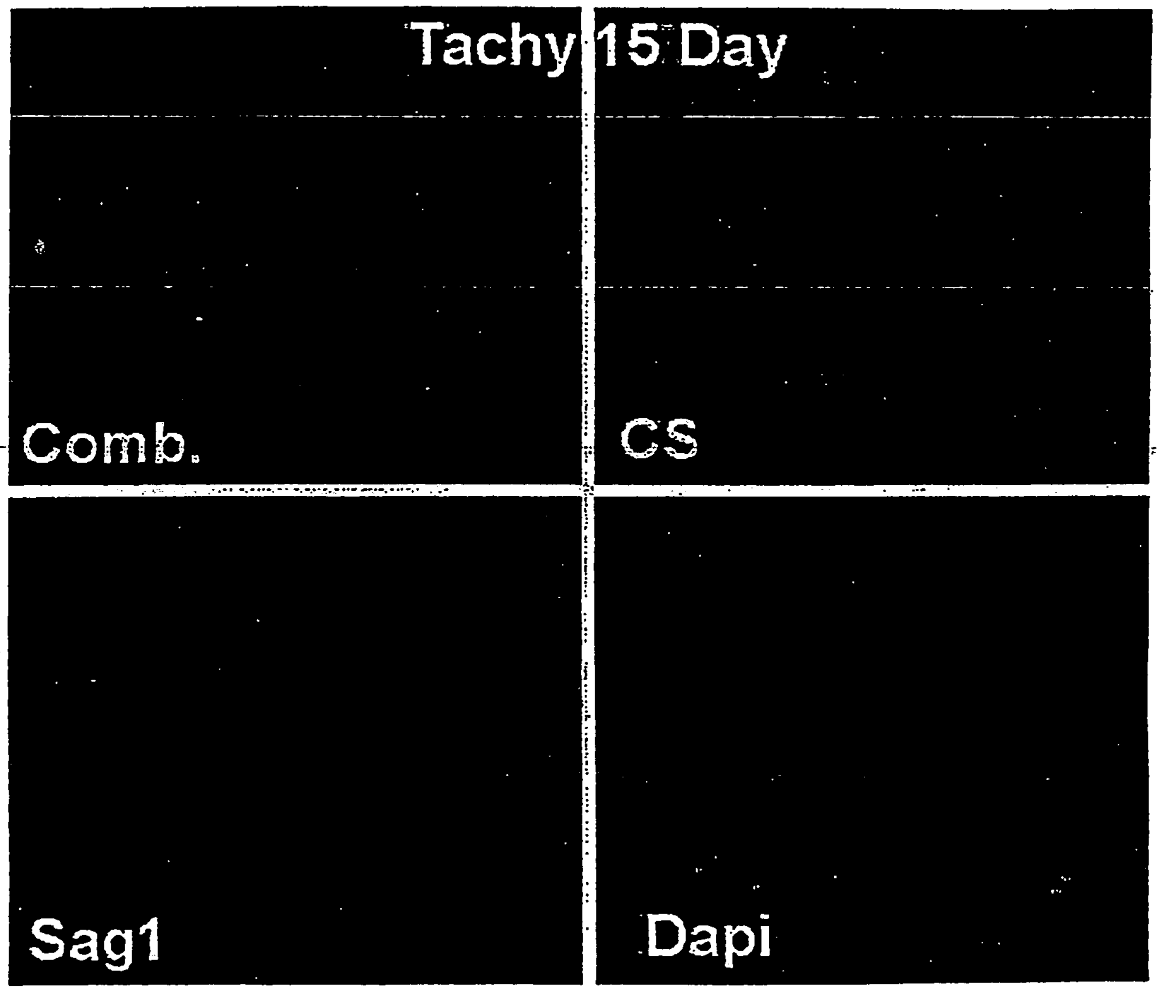

Fig 16A1

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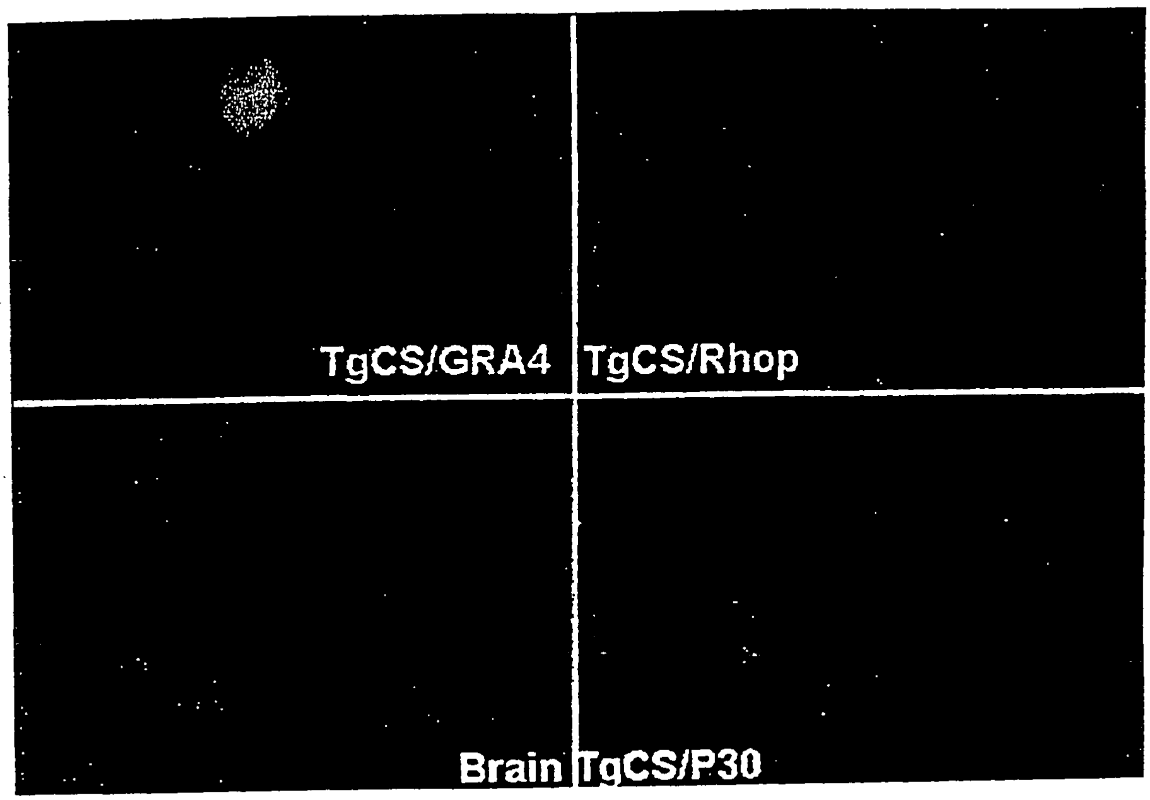

Fig 16A2

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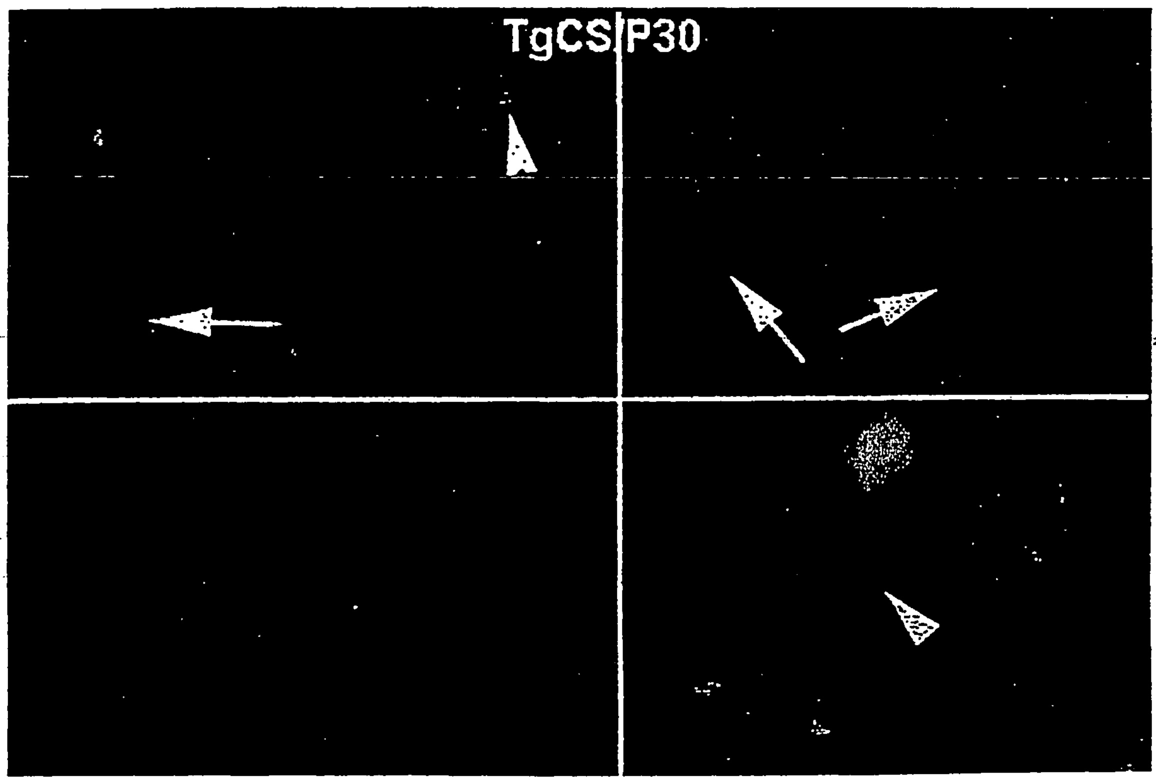

Fig 16A3

RECTIFIED SHEET (RULE 91)
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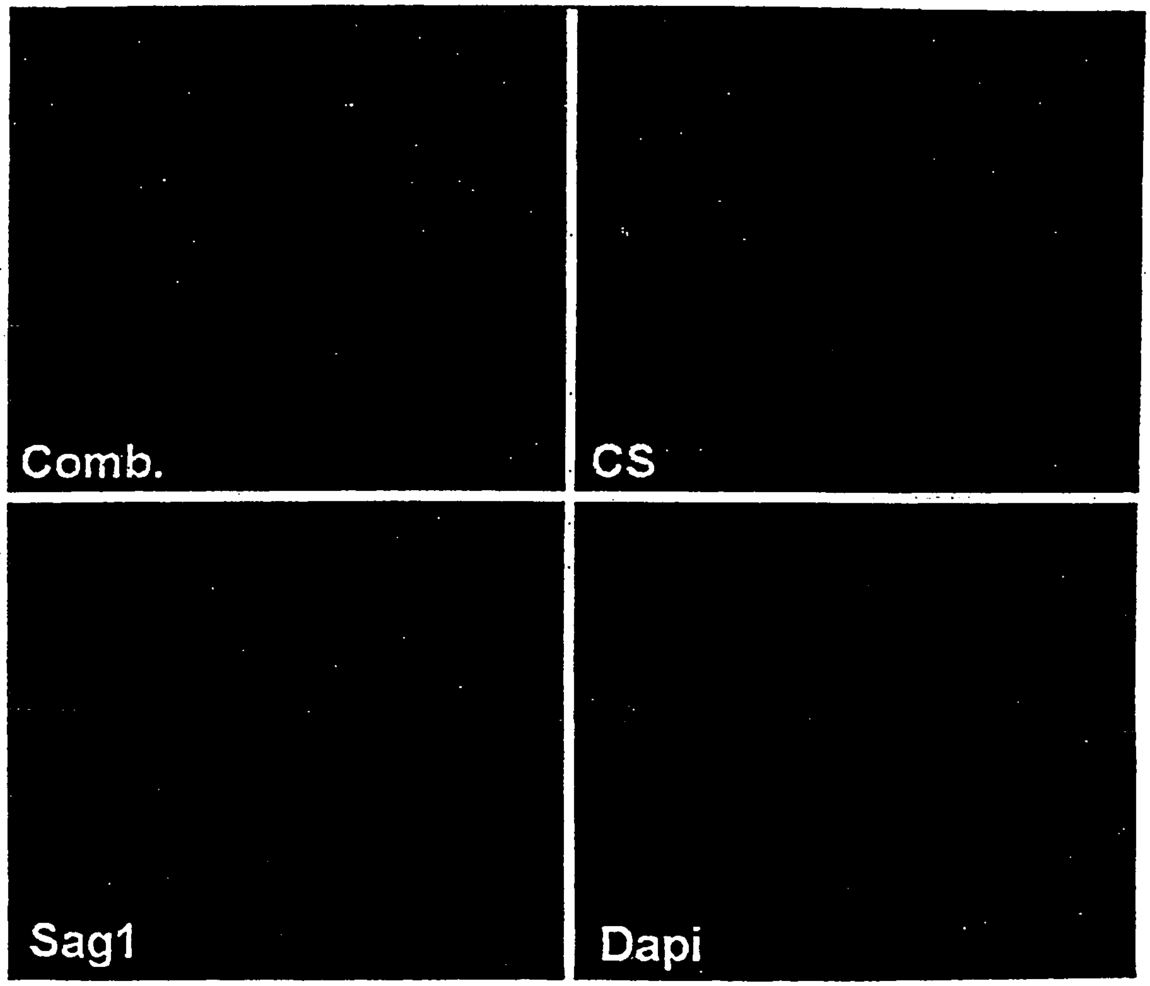

Fig 16B1

RECTIFIED SHEET (RULE 91)
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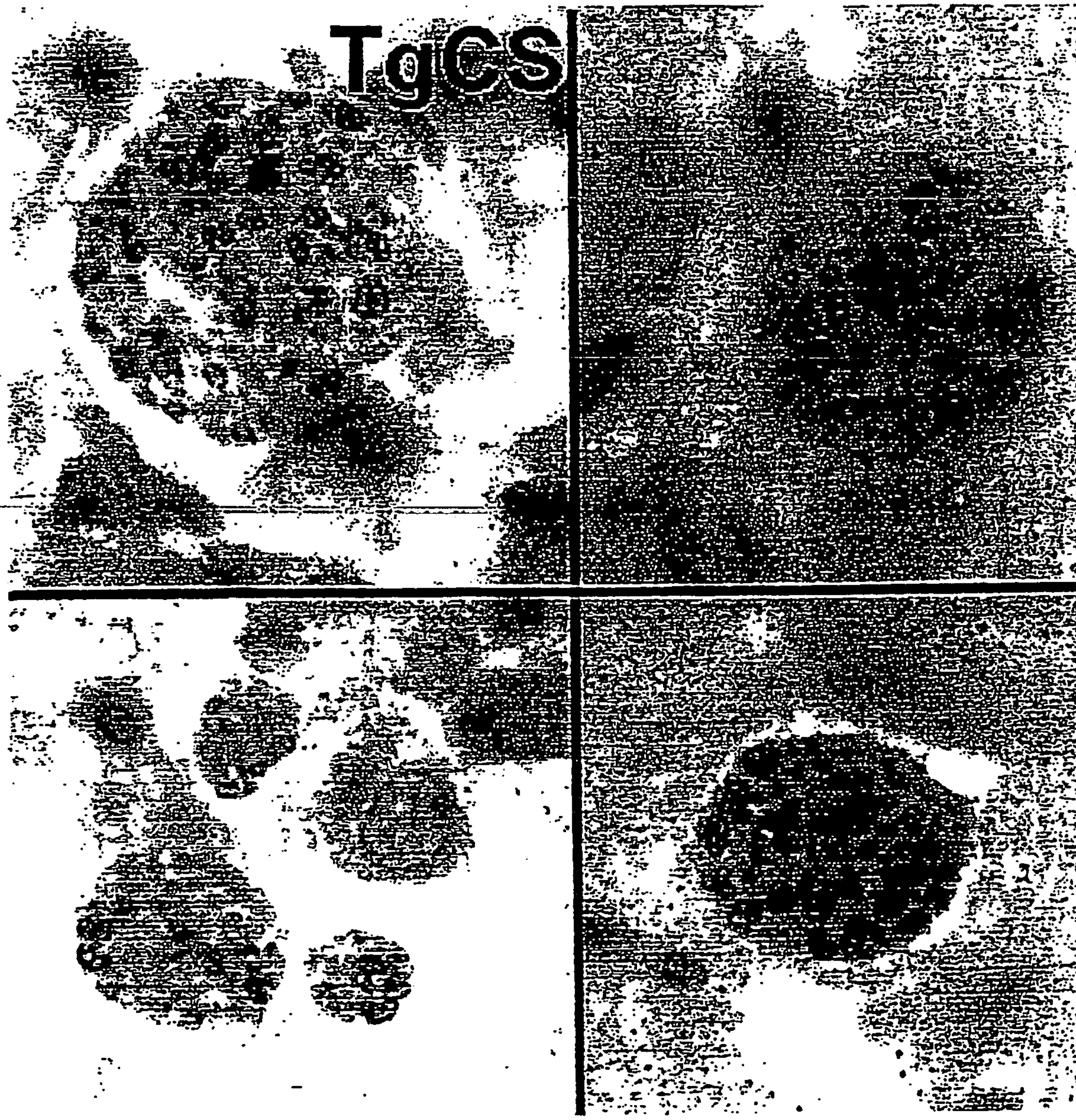

Fig 16B2

RECTIFIED SHEET (RULE 91)
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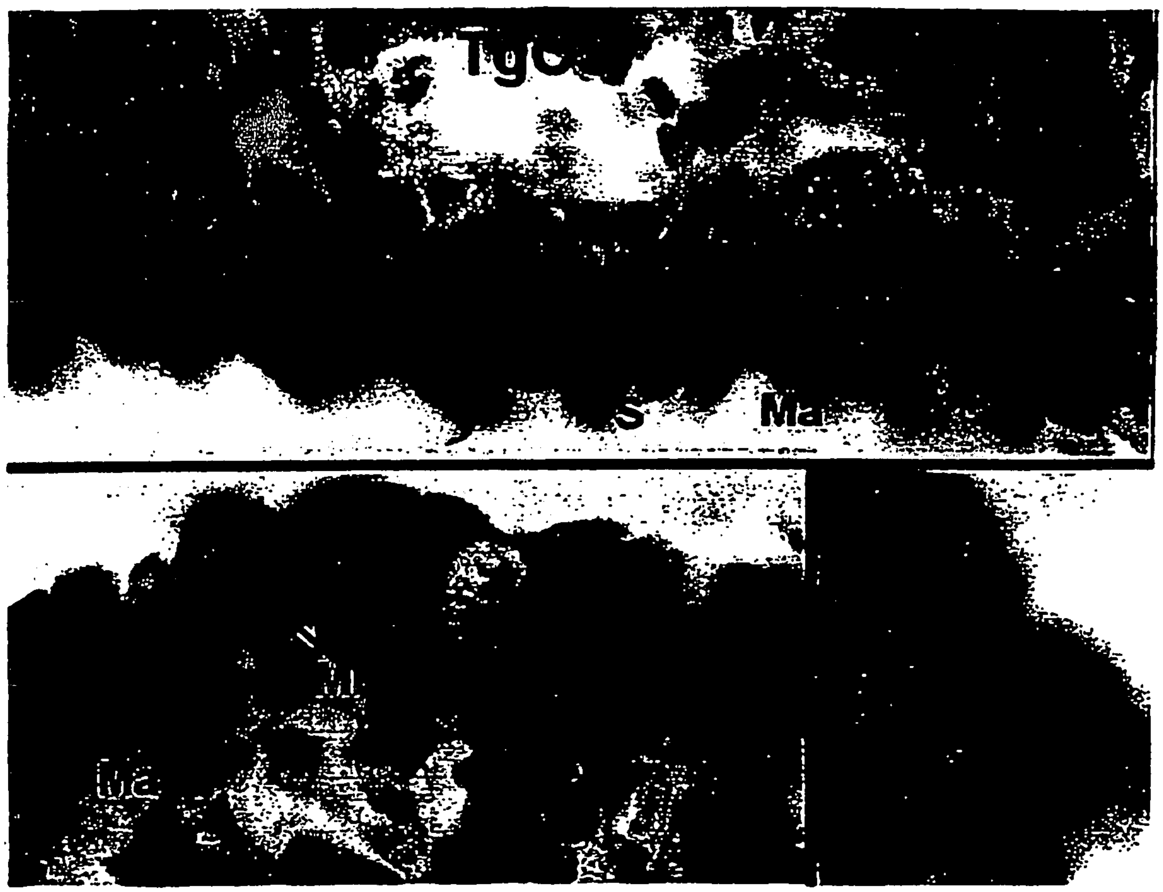

Fig 16C

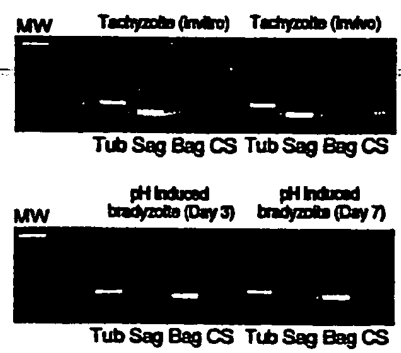

Fig 16D

RECTIFIED SHEET (RULE 91)
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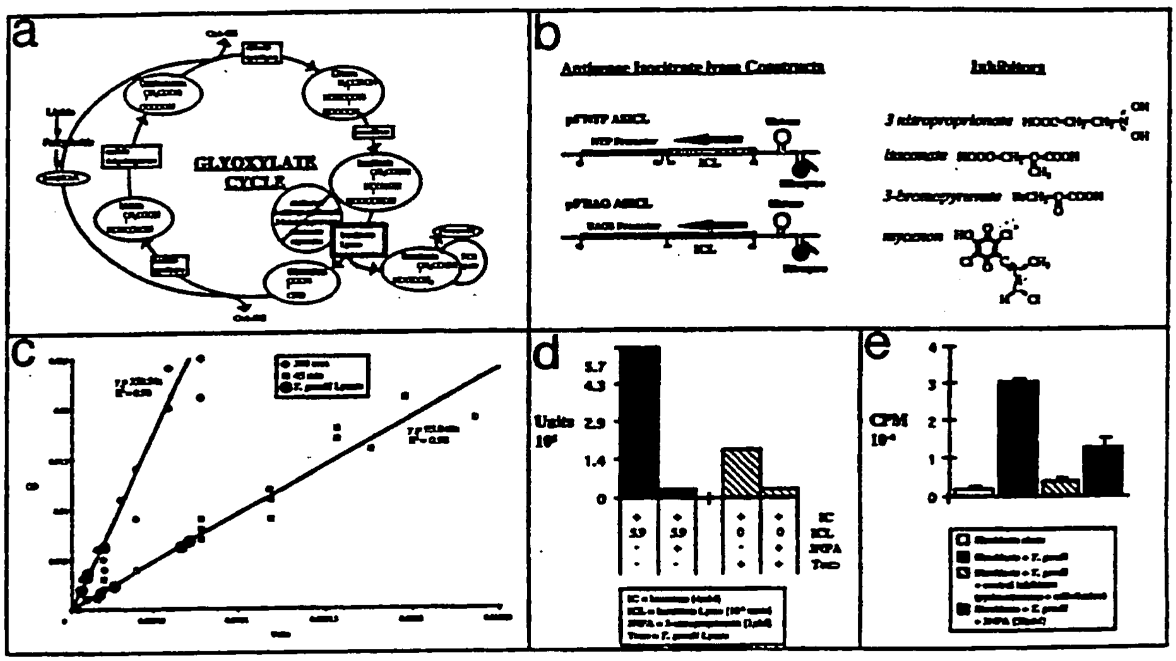

Fig 17

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Fig 18 ICL cDNA sequence

TgESTzz53...+3' PCR Fragment Translated Sequence Page 1
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Sequence Range: 1 to 1499

```
                10         20          30         40         50
AA TAC CCT CCG AGT TCT ATA CGT TTC TTC GGT TTT TGC TAA GCC ACA AAC
   Tyr Pro Pro Ser Ser Ile Arg Phe Phe Gly Phe Cys *** Ala Thr Asn>
   ___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

                60         70          80         90
TGC AGG CTT AGC AGG CCA CCT TCC GTC GTG AAC TCG TTC ACC GAG TTA
Cys Arg Leu Ser Arg Pro Pro Ser Val Val Asn Ser Phe Thr Glu Leu>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

100         110         120         130         140
CCG GCC TCA CAC CTA TTT TCG TTG CCG TTC TGG AAA GTC AGT AAG GGA
Pro Ala Ser His Leu Phe Ser Leu Pro Phe Trp Lys Val Ser Lys Gly>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

  150         160         170         180         190
CCA CCT TCA CGT GCA GTT GAC CGG TCT GCA ATG ACC ATT GAG TTC GAT
Pro Pro Ser Arg Ala Val Asp Arg Ser Ala Met Thr Ile Glu Phe Asp>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

      200         210         220         230         240
GTC CCG AAA TCC TTT TGT TTT GAT TTC CGC AAG GAG TGT CTT GAA CCA
Val Pro Lys Ser Phe Cys Phe Asp Phe Arg Lys Glu Cys Leu Glu Pro>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

      250         260         270         280         290
CTG TCC GTG TCT ACT TCC TTT TTC GTC GCG CTT CCG CGC CGT CTC CCC
Leu Ser Val Ser Thr Ser Phe Phe Val Ala Leu Pro Arg Arg Leu Pro>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

         300         310         320         330
GTC CTC GTC TCC GCC TTC CGT CTC ACA ACT TCC CTT CAT TCT CAC AGC
Val Leu Val Ser Ala Phe Arg Leu Thr Thr Ser Leu His Ser His Ser>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

340         350         360         370         380
ATG GCG TCT CGT GCT CCC CAT GCT GGA CAG CGC TTG CGC AGC CTC ATG
Met Ala Ser Arg Ala Pro His Ala Gly Gln Arg Leu Arg Ser Leu Met>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

  390         400         410         420         430
CAG AAG AAA TGC GTC ATG CTT CCT GGG GCT TAC AAC GGT CTC ACC GCG
Gln Lys Lys Cys Val Met Leu Pro Gly Ala Tyr Asn Gly Leu Thr Ala>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

    440         450         460         470         480
CGC CTC GCG GCT GAA GCA GGA TTT GAA GGA GTC TAC GTC TCT GGA GCT
Arg Leu Ala Ala Glu Ala Gly Phe Glu Gly Val Tyr Val Ser Gly Ala>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>
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        490         500         510         520         530
 *       *       *       *       *       *       *       *       *       *
GCT CTC AGT GCA TGC CAA GGC GTC CCC GAT ATC GGC ATA TTA GGT CTC
Ala Leu Ser Ala Cys Gln Gly Val Pro Asp Ile Gly Ile Leu Gly Leu>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

          540         550         560         570
    *       *       *       *       *       *       *       *       *
GAA GAC TTT ACT CGA GTA ATC TCC CAA GCC GCC TCT GTC ACC AGC CTC
Glu Asp Phe Thr Arg Val Ile Ser Gln Ala Ala Ser Val Thr Ser Leu>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

580         590         600         610         620
 *       *       *       *       *       *       *       *       *
CCT GTT CTC GCC GAT GCA GAC ACG GGG TTC GGT GGC CCT GAA ATG GTT
Pro Val Leu Ala Asp Ala Asp Thr Gly Phe Gly Gly Pro Glu Met Val>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

  630         640         650         660         670
   *       *       *       *       *       *       *       *
CGG CGC ACT GTC TTC GCG TAC AAC CAG GCG GGC GCG GCT GGG CTG CAC
Arg Arg Thr Val Phe Ala Tyr Asn Gln Ala Gly Ala Ala Gly Leu His>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

     680         690         700         710         720
 *     *       *       *       *       *       *       *       *
ATT GAG GAC CAG CGT TTG CCG AAG AAG TGC GGG CAT TTG GAG GGG AAG
Ile Glu Asp Gln Arg Leu Pro Lys Lys Cys Gly His Leu Glu Gly Lys>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

        730         740         750         760         770
 *       *       *       *       *       *       *       *       *       *
CAG TTG GTG TCC ATT GAA GAG ATG GAG GAG AAA ATC AAA GCG GCC GCT
Gln Leu Val Ser Ile Glu Glu Met Glu Glu Lys Ile Lys Ala Ala Ala>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

          780         790         800         810
    *       *       *       *       *       *       *       *       *
GCG GCG TCC CAG GAC TGC TCG AAC GGC GAC TTC ATC ATC TGC GCT CGC
Ala Ala Ser Gln Asp Cys Ser Asn Gly Asp Phe Ile Ile Cys Ala Arg>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

820         830         840         850         860
 *       *       *       *       *       *       *       *       *       *
ACG GAC GCC CGC AGT GTC GAC GGG CTT GAT GCG GCT GTG GAG CGA GCA
Thr Asp Ala Arg Ser Val Asp Gly Leu Asp Ala Ala Val Glu Arg Ala>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

  870         880         890         900         910
     *       *       *       *       *       *       *       *       *
GTC CGA TAC ACG GCA GCC GGA GCA GAC ATG CTT TTC CCC GAA GGA CTG
Val Arg Tyr Thr Ala Ala Gly Ala Asp Met Leu Phe Pro Glu Gly Leu>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

     920         930         940         950         960
 *     *       *       *       *       *       *       *       *       *
GAG ACA GAG GTG AGA GGT GGA AAG AAG AAT CAG AGG AAG AAG GCG TCT
Glu Thr Glu Val Arg Gly Gly Lys Lys Asn Gln Arg Lys Lys Ala Ser>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

        970         980         990        1000        1010
 *       *       *       *       *       *       *       *       *       *
```

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TgESTzz53...+3' PCR fra[illegible]ent Translated Sequence	Page 3
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GTA TTG GAG AGG CAG CGA GAG GCA GTC GCT CTG GAA GAG TTT CAA GCA
Val Leu Glu Arg Gln Arg Glu Ala Val Ala Leu Glu Glu Phe Gln Ala>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

        1020        1030        1040        1050
TTT GCG CAT GCA TTG GCG GTT TTG CCT GGC AAA GCG CCT TTC GGG GGG
Phe Ala His Ala Leu Ala Val Leu Pro Gly Lys Ala Pro Phe Gly Gly>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

1060        1070        1080        1090        1100
CCC TAT CTG CTC GCA AAT ATG ACG GAA TTT GGA AAG ACG CCC ATC ATG
Pro Tyr Leu Leu Ala Asn Met Thr Glu Phe Gly Lys Thr Pro Ile Met>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

 1110        1120        1130        1140        1150
GAG CTT TCC ACC TTC GAA GGC CTT GGA TAC CAC TGC GTT ATC TAC CCT
Glu Leu Ser Thr Phe Glu Gly Leu Gly Tyr His Cys Val Ile Tyr Pro>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

   1160        1170        1180        1190        1200
GTT TCA CCT CTC AGA GTC GCC ATG AAA AGC GTC AAG GGC ATG CTG GTC
Val Ser Pro Leu Arg Val Ala Met Lys Ser Val Lys Gly Met Leu Val>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

     1210        1220        1230        1240        1250
GAC TTA CGC AAG AAT GGC AGC GTT GGC CAT AGC CTG GAG AAA ATG TAT
Asp Leu Arg Lys Asn Gly Ser Val Gly His Ser Leu Glu Lys Met Tyr>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

        1260        1270        1280        1290
ACA CGG CAG GAG CTT TAT TCC ACT CTG CAC TAT CGG CCG GAA GGG ACG
Thr Arg Gln Glu Leu Tyr Ser Thr Leu His Tyr Arg Pro Glu Gly Thr>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

1300        1310        1320        1330        1340
TGG ACG TAT CCC TCA GCG AGT GTG TGC ATG GAC AAA GCC GTG GAA GAT
Trp Thr Tyr Pro Ser Ala Ser Val Cys Met Asp Lys Ala Val Glu Asp>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

 1350        1360        1370        1380        1390
ACC GAG GCC TAG GGA GTC TCA GGC TCG GCA TTT TCT TTT TCT CGA CTG
Thr Glu Ala *** Gly Val Ser Gly Ser Ala Phe Ser Phe Ser Arg Leu>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

   1400        1410        1420        1430        1440
GTC TCA CCA ATA CAA AAG ACA ATG CTC ACA GAC GAA AAG CAG AAG TTC
Val Ser Pro Ile Gln Lys Thr Met Leu Thr Asp Glu Lys Gln Lys Phe>
___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

      1450        1460        1470        1480        1490
TGA TTG TAT TTA TGA AAC GTG AAA AAA AAA AAA AAA AAC TCG AGG GGG
*** Leu Tyr Leu *** Asn Val Lys Lys Lys Lys Lys Asn Ser Arg Gly>
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TgESTzz53...+3' PCR fragment Translated Sequence Page 4
Thursday, April 22, 1999 3:52 PM

___a___TRANSLATION OF TGESTZZ53...+3' PCR FRAGMENT [A]_a___a___>

```
     *
GGC CCG GTA
Gly Pro Val>
___a___a___>
```

Translation of TgES1-+53...+3' Fig.19 ICL amino acid sequence Page 1
Thursday, April 22, 1999 3:52 PM

10 20 30 40 50

YPPSSIRFFG FC*ATNCRLS RPPSVVNSFT ELPASHLFSL PFWKVSKGPP

60 70 80 90 100

SRAVDRSAMT IEFDVPKSFC FDFRKECLEP LSVSTSFFVA LPRRLPVLVS

110 120 130 140 150

AFRLTTSLHS HSMASRAPHA GQRLRSLMQK KCVMLPGAYN GLTARLAAEA

160 170 180 190 200

GFEGVYVSGA ALSACQGVPD IGILGLEDFT RVISQAASVT SLPVLADADT

210 220 230 240 250

GFGGPEMVRR TVFAYNQAGA AGLHIEDQRL PKKCGHLEGK QLVSIEEMEE

260 270 280 290 300

KIKAAAAASQ DCSNGDFIIC ARTDARSVDG LDAAVERAVR YTAAGADMLF

310 320 330 340 350

PEGLETEVRG GKKNQRKKAS VLERQREAVA LEEFQAFAHA LAVLPGKAPF

(X)

360 370 380 390 400

GGPYLLANMT EFGKTPIMEL STFEGLGYHC VIYPVSPLRV AMKSVKGMLV

410 420 430 440 450

DLRKNGSVGH SLEKMYTRQE LYSTLHYRPE GTWTYPSASV CMDKAVEDTE

460 470 480 490

A*GVSGSAFS FSRLVSPIQK TMLTDEKQKF *LYL*NVKKK KKNSRGGPV

RECTIFIED SHEET (RULE 91)
ISA/EP

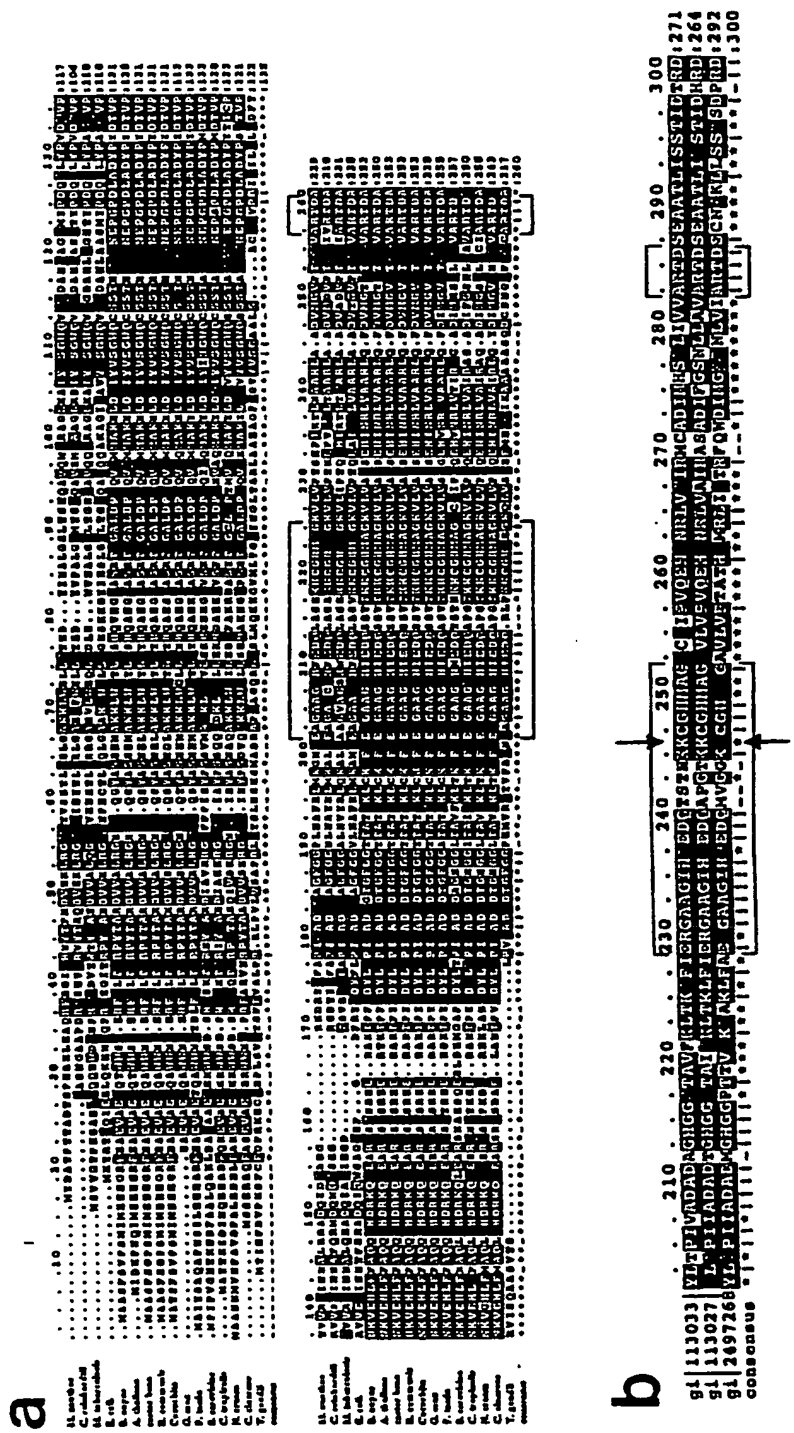

Fig. 20

CCCTATTACGTTTCCTTTTTTTAAATGCGGCGAAAACATTCCCTCCATAC 050
AGATTTCCCATTCACGTGACGTCTCGCGTGTTTCAAACGTCAACTGGTTT 100
TCCCTGCTCTTGTAGTCACAAGACCGTGCAACCAAACCTGCGACACAATC 150
TTGTGCCTGTGACCACCGCACCGCAACTGCCCACTCTGTAAACATAGTCC 200
CTCCCTAAACCGTCAAAACCCCGAAACGAACCGGATGCTCTTCTCTCGTC 250
CTTTCTCCCTCGTTTTCCTTTCTTAGAAAACAGGAAAAATCCTCACTGGA 300
TATGTGCACATTTACCGAAGCGATGCGGAATCCACGGCGAGGTGGCGGGT 350
CAACTCCCTTGGCCAGGGGTTGAGTCTGGTAGTGGCATTTTTAGGCGTAG 400
AGACAATGTAAAGGTCTCCCATTGAACAGAACCTGCTTACTCCTTCGTCT 450
TAGCCCCTCAATTCTGCATTTACAATCCCTTTCAAAAGCAACAAAGTCTT 500
ACATCCAAAACCCTCCAAAATCCCGTGGTGTGTGACCTTTCCAGTGACTC 550
TTGCTCCCACAACCGTGCGCCCTTTTTCGCGGCTTGCCGAAACATCGAAA 600
AGCTGCGTCGCTCGCATTACTGCTTTTTGGGCCTTCACTTTTCCCCAAAT 650
ACCCTCCGAGTTCTATACGTTTCTTCGGTTTTTGCTAAGCCACAAACTGC 700
AGGCTTAGCAGGCCACCTTCCGTCGTGAACTCGTTCACCGAGTTACCGGC 750
CTCACACCTATTTTCGTTGCCGTTCTGGAAAGTCAGTAAGGGACCACCTT 800
CACGTGCAGTTGACCGGTCTGCAATGACCATTGAGTTCGATGTCCCGAAA 850
TCCTTTTGTTTTGATTTCCGCAAGGAGTGTCTTGAACCACTGTCCGTGTC 900
TACTTCCTTTTTCGTCGCGCTTCCGCGCCGTCTCCCCGTCCTCGTCTCCG 950
CCTTCCGTCTCACAACTTCCCTTCATTCTCACAGGTGGTGTACTGCAATC 1000
ATAAAGAACTTGGCTGTCTGCACCTCTTATGCAGAGTCATATTCAGTCTC 1050
CTACGGAATATCATGTCCACAAATAAAGAAAACTGGTTTGATTGTATCTC 1100
ATCACTGACTGTCGTCCGACCCTTCCCCCCCATAAAATAGCTGCTAACG 1150
TGCAATGATTCGAGATACATTTATCTACCGCACTTTAGTTTAATACCCCG 1200
GTTTGTGGTTAGGGTTGTATGAACGCAGGAATACTTGTAGATCTTTGGAG 1250
CTTAAATATAAAAGATGCATGTTTATATGTGAATCTTTCAATGAAAACAT 1300
GTACGTGCATCTACACGTCTTGAAACGTAGGTGTACAACAATGTGCTTGG 1350
GAAGTCACTGCCTCTCTACAAATCACATAGTTTCTGTACGGTGGCGCCTC 1400
ATTTTCTTTCTTTGACTCTCTGTTTGCGTGTCAACATGATCTACCCTCGA 1450
TCCTCCCAACAGTCCTTTCGCTGTGCTTATCACTCTTTTTCTTTCAGTCC 1500
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CTTCTTCCCTGACGTGGTCTTGTTGCGGTTGTCCGGGTTTCCCTCTGTCA 1600
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TGCTCCCCATGCTGGACAGCGCTTGCGCAGCCTCATGCAGAAGAAATGCG 1700
TCATGCTTCCTGGGGCTTACAACGGTCTCACCGCGCGCCTCGCGGCTGAA 1750
GCAGGATTTGAAGGAGTCTACGTCTCTGGAGCTGCTCTCAGTGCATGCCA 1800
AGGCGTCCCCGATATCGGCATATTAGGTCTCGAAGACTTTACTCGAGTAA 1850
TCTCCCAAGCCGCCTCTGTCACCAGCCTCCCTGTTCTCGCCGGTGCGTAG 1900
CAGAATCGTGTTCTTCACTTCTTACTTCTATCTGCTTTGTGTCTTTCCTG 1950
TTTTTGGTTCGACTTGCTTGTCGATGGATAGAACCCCACGTTGGGTGTTC 2000
CGACGCGCCTCGAGCTTCTTCAGTTGCCCTACCTTCTGTACTCTTCCTGA 2050
CTTCGCTTCCTAGTCTCGAGGATCCACGTCGCTTTTCGACTCGTCCCTTG 2100
TCGCCGTCATCGCTTCAGAAACCGTTCACATCTACTGGCCCTTCCTCGTC 2150
TTTTCTTTTCCTCGATGTCCTTTTCCCAACTTTTCGCTCTGCTCTCTCTC 2200

FIG. 21

TCCTCTGTCGACGGTCTGGTCACTCATTCGTTTCGTGTCGCGTTCCCGTT 2250
GTGCTCTTTTCTCTCTTCTTCTCGTCCCTCTCCGTCTTCTCGCTCTCCTG 2300
TTCTCCTACCCGCTCTCCTTTTCTGTCTCGTCCGCTCAACCTCTCTCTCT 2350
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GGTTCGGCGCACTGTCTTCGCGTACAACCAGGCGGGCGCGGCTGGGCTGC 2450
ACATTGAGGACCAGCGTTTGCCGAAGAAGTGCGGGCATTTGGAGGGGAAG 2500
CAGTTGGTGTCCATTGAAGAGATGGAGGAGAAAATCAAAGCGGCCGCTGC 2550
GGCGTCCCAGGACTGCTCGAACGGCGACTTCATCATCTGCGCTCGCACGG 2600
ACGCCCGCAGTGTCGACGGTGGGTGACCCTCGAAACGGCCGAAAACAGAA 2650
CTCTAGGGTCTCGCGCATTCAGCGCGGGTGTCCCCTCGAATGGACGCTAC 2700
AGTGCTGTTAGTGTCGAGTGTTTTTAGCGACTTTCTTCAGAGCTCACTTA 2750
GGTTTCGTACGATTTCAATCGACAGACGGAAAGACGCTCAAGTGAAATTC 2800
GGGCCACCGAGAAGGCGAAGAGAGAGCAGAGGAAGGGAGGACCGGGAACC 2850
TTTGGACTACTGAGAAGCAGGCGAAGACGGGCGTTTCAGAAGCGCCTGAG 2900
CAGGTCTCCACACCGAGAGAAGCAGACTGAAGACGCAGTTCAGATGAAGC 2950
TCGAAAACCGGAAAGCGCCTCTTTAATATTGTAGAGGGAGTCTTAAGTCG 3000
TGCCTCTTTTCTCCCTGTCTTTCTCGCTGTCTCTGCATGGCTCAGGGCTT 3050
GATGCGGCTGTGGAGCGAGCAGTCCGATACACGGCAGCCGGAGCAGACAT 3100
GCTTTTCCCCGAAGGACTGGAGACAGAGGTGAGAGGTGGAAAGAAGAATC 3150
AGAGGAAGAAGGCGTCGTATTGGAGAGGCAGCGAGAGGCAGTCGCTCTGG 3200
TGAGAAGCTGCGGCGGAAAGGGAGAAAGAAAAGAAATGAAAAAACCCGGT 3250
CGAGAGGGATGGAACTCTGAAAACTCGGAGAAGTGGAGAAAGGGAGCTAG 3300
GAGCAGAGGAGGTGAAGGAATCCGTATAGTGGATTGATGTGTGACGTCAA 3350
CTATGAAAGACATGACAAATTCAACTACAGGCGAAGGGTATGACAGGGAC 3400
ATGCGTTTTGTACAGAAAACAGAGGACAATGAACATGTCAGACCTCATAC 3450
CACACGCGAAGAGATGCGCAGTGGATTATGGAATGAGCAAGAGTAAGGAG 3500
TGAAACTTCACAATGTGCATTCGGTGTCAGATTGAGTCATCAAATCTCGG 3550
TGTTCGTGCTCTTTTTTCTCGTCTGCCTCCAAAAGTGTGTCCTTGCCTTC 3600
CTCATGTCTGCTCTGCACCCATTGTCCTTCACCGTGTTCCGTTCGCTCCC 3650
CGTATGCCTGCGGTTTCTTGTCCGTTATCAGTCTCTACCGGGTTCATCTC 3700
CTCTTTCTGCGGAGAGGCTTTTGTTCTAGCGATGGGTGTATGAGTTCGTT 3750
TCTGTCATCCTCATATACTACCGTCACGAGACAAACAACTGCTCCATGGT 3800
CGCTGTACACGGCCAACTTGTTGGGCTGCTCACAAAAGCCACAAGTGTCG 3850
AGTTTCAAAATTCAACCACATTAGTGTTGTTCCACGTCGGTTACGTTTAC 3900
GCGTTTCGCGAAGAAGACGAAGACGAAAGACGCGTCCATTTCAGAGAAGA 3950
CCTGTCCGTTTTCGTTGTGACACCAGGAAGAGTTTCAAGCATTTGCGCAT 4000
GCATTGGCGGTTTTGCCTGGCAAAGCGCCTTTCGGGGGGCCCTATCTGCT 4050
CGCAAATATGACGGAATTTGGAAAGACGCCCATCATGGAGCTTTCCACCT 4100
TCGAAGGCCTTGGATACCACTGCGTTATCTACCCTGTTTCACCTCTCAGA 4150
GTCGCCATGAAAAGCGTCAAGGTACGTTTGTCCTGCTATCCATACTGAGT 4200
GACTCGGATCGATTTCTTCGTTTGCTGTGGCACGTGGAACTGAGTGCCAT 4250
ATGCGTGTACGCAAATGCAGAGGAATGCATGCATGTGAGCACACCTGTCT 4300
GCAGCTACGCGAATCTCTGCCTGTGTTGACCTTCTACCTGATGGCAGGCA 4350
TGCACGTGTATACACGCACAAGCATCTGTATAAATATGTGTAGTTGAGTA 4400

FIG. 21

ATTATACGTGACCTATTAAATCTAAAGCAGAAAACATGCTCATACCGTTC 4450
TTGTTGTTGCTCAGGGCATGCTGGTCGACTTACGCAAGAATGGCAGCGTT 4500
GGCCATAGCCTGGAGAAAATGTATACACGGCAGGTACAGCGTTACCATCA 4550
TAAGGCGGATACTTATAAGATTTTCCTTCAATGACGTGCATGCATCACGG 4600
ATACCAAACCTGCTCGTTTAATCCTCTGTTTTGCTCTGTAAGCGTCTTCC 4650
TTCTTGTATTCTTCCATCCTTTCATCTGCCGTTGTGTCAATTTCTGCCCT 4700
GGGGCTCTGTCTTCGCTTTAATGCCCTCAGTGTTTTTCTTCTTTCTTGCC 4750
TCTCCTTATTCTGTCTCACGGTTCCTGTTTGTCTTCTGGTATCTCGTGCT 4800
GTTCGTGCTTTTAGGAGCTTTATTCCACTCTGCACTATCGGCCGGAAGGG 4850
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TCTCACCAATACAAAAGACAATGCTCACAGACGAAAAGCAGAAGTTCTGA 5000
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CCTTTGGTGTATGAATGCATGGTCTTTTTCAATTCCATCTGGCTGCTTCC 5150
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AGAAAACCGAAGAGGATGGACAACTTACCCCACCGAGGACAGACCACAGA 5350
TGCGAAAAAGAGAATGAATCGAGAGAAAAGAAATGCGAGCCGATGCAGAG 5400
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GGAAACCGGAAAGTGGACAAAACTCCTTTAAAATGCGGAAGAGTGAGGCG 5500
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AACCCAGTAGAGTGCTCGTGACATCTTCCACTTTCGTGTCCTCACTTGGG 5600
TGCTCGGTTTCTGCAGTGCAAGCTGCTTCTCGCTGTCCTCACTTCTTTCT 5650
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ACTCGCACTACCCTCCGCCGCGACTGCGTCTTTTTCTCCACTCTGCGAAT 5950
CTCACTTTTCTTCTGAATTTCTTTGTCGACGAGGAACCGACCGCGTAGAC 6000
GGCGGCACAGCGTTTCTAGCAGATATTCGGGTTTTGTGTGATTAGTGTCT 6050
GTCTCTTTCTCTCACTCTCACTTCTTGCCCGGGAAGGAGGAACGCCGCAG 6100
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AAGATATCCTTGCGATGAGCACGCAAAGAAGCCTGGAACGCATGCGCTAG 6300
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ACCGCGATGACCACGGGAAACGGACAGACTCTACAAACCTCCAAAATCT 6400
CTGTCCGACACCAAAAAAACAAACACGGATTCCCGACGACAAAAAGACTC 6450
TCAACATCACATCCATGTGTGCATCTCTCTACACACTTGTGGCGGAATAC 6500
ACATTTGTATCCATACATATACTTTCTAGTCGCGCTGCAGAGAGCTCCGT 6550
CGGTGTTCCTTCCTTGATCGGAATGGCCTCGCTAGCGAGAGTCTTTGCCA 6600

FIG. 21

```
TTTCGCCACTTTTCCCTCTCTAGTTCAAGGTCTGAAAAAGACCATTTACG    6650
TTTTGAACTCTGCTCTGTCTCTCGGATCGCTCATCTGCTTTCCAGCTCCC    6700
TCTCTCCGCACATAAGCCGAATGTCATTCTCTCCTCTCAGTCTGCCCTTG    6750
CCCGGCTTCCCAGACGAGGGGTTTTACGAAAAAATGCCGCCTCACCGTCA    6800
GAGCATTTGCTCCACACCTTCTTCCGCTGGCTTTCCCCTCTGCTTCTCCC    6850
GTGTTTCTCTTGATTCACTTTTGCGTTTCTCTCTTGTCTCCGCCCCGTCG    6900
CGCGACCGCTTCAATCTAGGAGAGGCACACTCCCCCCGAAAGAGCGTGTT    6950
GCTTTGCGCCTTCTCCTTCTAACTCGCTTTCCCCACAGGAGGCAGTTAAG    7000
AAGAATCTCAAAAGGATCCCAGAAGACACCCTTAGAAATCTCGAAAAAAC    7050
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FIG. 21

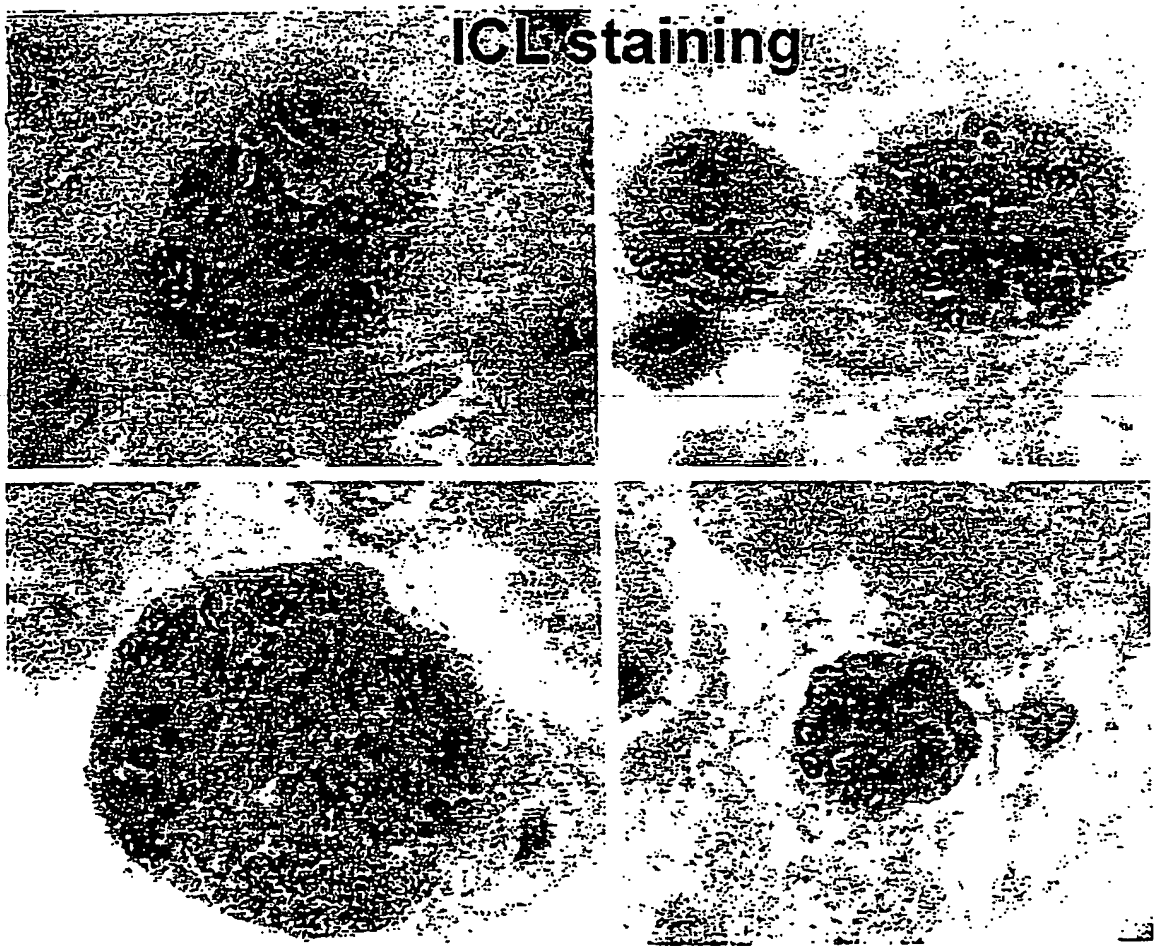

Fig 22

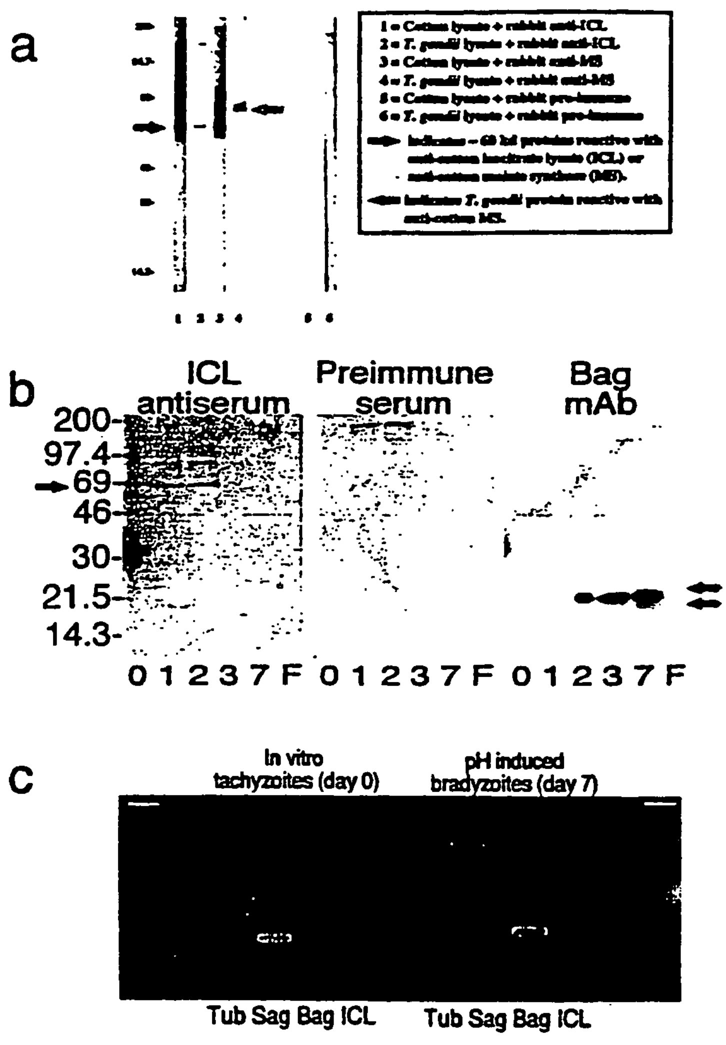
a
b
ICL antiserum
Preimmune serum
Bag mAb
200-
97.4-
69-
46-
30-
21.5-
14.3-
0 1 2 3 7 F 0 1 2 3 7 F 0 1 2 3 7 F
c
In vitro tachyzoites (day 0)
pH induced bradyzoites (day 7)
Tub Sag Bag ICL
Tub Sag Bag ICL

Fig 23

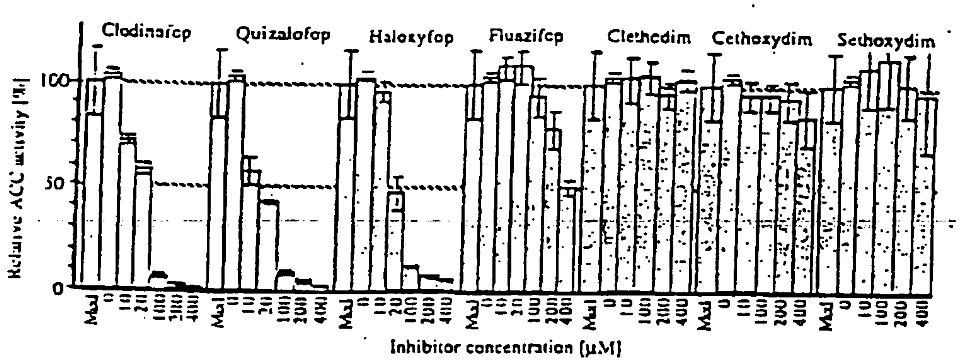

Fig 24A

RECTIFIED SHEET (RULE 91)
ISA/EP

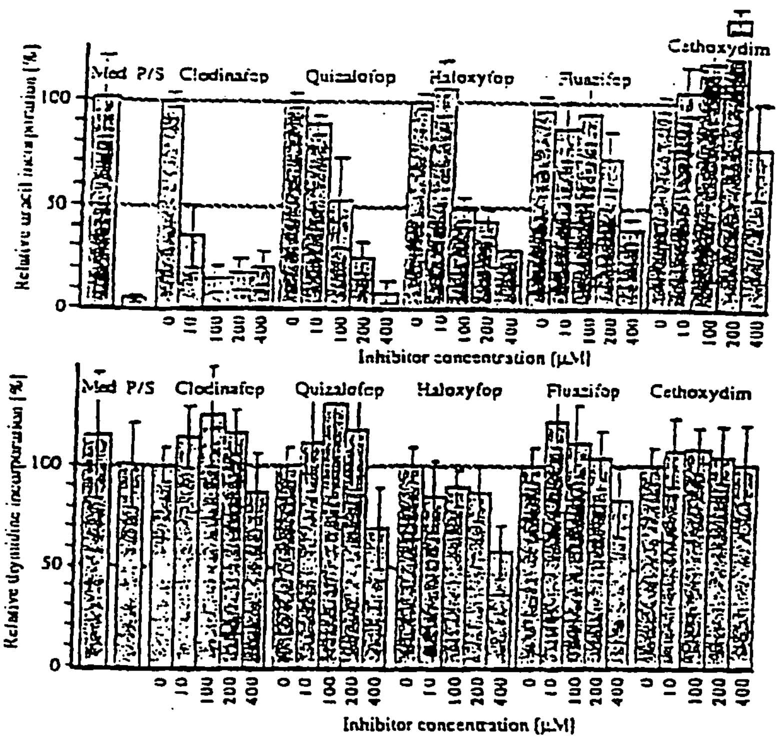
Relative uracil incorporation (%)
Med P/S Clodinafop Quizalofop Haloxyfop Fluazifop Cethoxydim
0 50 100
0 10 100 200 400
Inhibitor concentration (μM)
Relative thymidine incorporation (%)
Med P/S Clodinafop Quizalofop Haloxyfop Fluazifop Cethoxydim
0 50 100
0 10 100 200 400
Inhibitor concentration (μM)

Fig 24 B

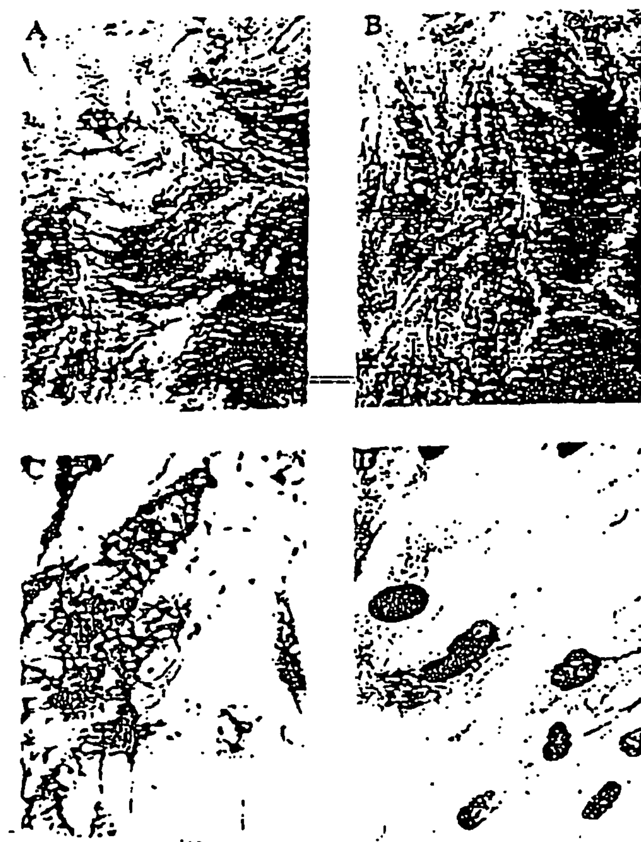

Fig 24C

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GenBank files:

LOCUS       AF157612     5258 bp    DNA             INV       30-OCT-1999
DEFINITION  Toxoplasma gondii acetyl-CoA carboxylase 1 (ACC1) gene, partial
            cds.
ACCESSION   AF157612
KEYWORDS    .
SOURCE      Toxoplasma gondii.
  ORGANISM  Toxoplasma gondii
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eimeriida;
            Sarcocystidae; Toxoplasma.
REFERENCE   1  (bases 1 to 5258)
  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Growth of Toxoplasma gondii is inhibited by
            aryloxyphenoxypropionate herbicides targeting acetyl-CoA
            carboxylase
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 5258)
  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-JUN-1999) Molecular Genetics and Cell Biology,
            University of Chicago, 920 East 58th Street, Chicago, IL 60637, USA
FEATURES             Location/Qualifiers
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                     PAAQLQVAMGIPLWRIPDIRRFFGRDPHAGDRIDFINEDYLPIQRHVLASRVTAENPD
                     EGFKPTSGRVDRLEFQPLENVWGYFSVCASGGVHEYADSQFGHIFATGKNREEARKKL
                     VLGLKRVDVRGEIRTPIEYLVQLLEDKDFIESRIDTSWL"
BASE COUNT     1176 a   1271 c   1351 g   1460 t
ORIGIN
        1 cgcgtcctca tcgccaacaa cggcatggca gccaccaagt cgatcttctc catgcgtcag
       61 tgggcctaca tggaactcgg cgacgacaag gtgagcctga cacagtgaac aaggtggatc
      121 tcttgttagc tttcgaaatg ccatatctct aaaatgttga agagctgacc tgacgcaaag
      181 ctaaatattc atgaagactc tcttgtcacc gttagtggat tccgtttttg tcttgccccg
      241 ctctctatct tgtttttcgc cgcaacagag aactgtaact gtatatacag tgatatatat
      301 agttatatgt acgtgttttt tatgcgcgta tgtgttcagt cacaactaca aaataaatgt
      361 acacgtacat gcttagatag ttacgtggcg acaaacctct tctgtgtcag ctatgcgaat
      421 cgcgcgaaaa ggcgaccgag acatgaagct ctcttccttc gcatttctag catttgcata
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Fig 24 · d pag 1

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1321 cctcactggg tctccattga ttgaacaaaa ctttatgcat tttctttcaa ctcgctcttc
1381 tttcctctcg gcatcgtttt gccaggacct cctgttcttt caagaaacac gcggcaggga
1441 ggcatttgat ggatcactat gtcggttgat gatgtttgtg aagagtactt gccgcgttac
1501 tgtacaacct ctatcgtaca tgttagagga gaaaacggat cttttttttgg aggtacccgc
1561 tcctcgaaat ctagactgtc atccgatttc tagggcgtgg ttagtgaagc acgcgcgcgt
1621 ctttgtcggtt gtctctgatt ctgtttttttg gcaagacgat ggaggatgaa cagaggaatt
1681 ttttgtcact accactgacg agccgagagc tcgatgattg gactgttccc tcgagtaaat
1741 ctgacgcgtc gtctttatag cgtttcgtct ctgaagcgat tcgtcctact ctttctaggta
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2401 ctgcattttt atttccactc tgtttttgcg tctttcctta ggtggacgca gtgtggccgg
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2581 tcctcgcgca gacagcaggc gttccgagca ttcccttggag tggagattct ctcaaggcga
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2761 ctctataagc agaaacgcat gaacggacaa aacgaacgtg aagaccttag acagggtaac
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Fig 24 d page 2

RECTIFIED SHEET (RULE 91)
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Fig 24 d page 3

RECTIFIED SHEET (RULE 91)
ISA/EP

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  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Growth of Toxoplasma gondii is inhibited by
            aryloxyphenoxypropionate herbicides targeting acetyl-CoA
            carboxylase
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 6965)
  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-JUN-1999) Molecular Genetics and Cell Biology,
            University of Chicago, 920 East 58th Street, Chicago, IL 60637, USA
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RECTIFIED SHEET (RULE 91)
ISA/EP

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Fig 24 d page 6

RECTIFIED SHEET (RULE 91)
ISA/EP

51/55

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SOURCE      Cryptosporidium parvum.
  ORGANISM  Cryptosporidium parvum
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eimeriida;
            Cryptosporidiidae; Cryptosporidium.
REFERENCE   1  (bases 1 to 393)
  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Growth of Toxoplasma gondii is inhibited by
            aryloxyphenoxypropionate herbicides targeting acetyl-CoA
            carboxylase
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 393)
  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-JUN-1999) Molecular Genetics and Cell Biology,
            University of Chicago, 920 East 58th Street, Chicago, IL 60637, USA
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Fig 24: d page 7

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DEFINITION  Plasmodium falciparum acetyl-CoA carboxylase 1 (ACC1) gene, partial
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ACCESSION   AF157615
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SOURCE      malaria parasite P. falciparum.
  ORGANISM  Plasmodium falciparum
            Eukaryota; Alveolata; Apicomplexa; Haemosporida; Plasmodium.
REFERENCE   1  (bases 1 to 393)
  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Growth of Toxoplasma gondii is inhibited by
            aryloxyphenoxypropionate herbicides targeting acetyl-CoA
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  JOURNAL   Unpublished
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  TITLE     Direct Submission
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     CDS             <1..>393
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                     IKYRGAGTIEYLYDQINKKYFFLKLNPRL"
BASE COUNT      156 a     39 c     72 g    126 t
ORIGIN
        1 tcacaaggtg gtggtgggaa aggtattcga aaagtggaga atgaatatga aataaaaaaa
       61 gcatatgaac aagtacaaaa tgaattacct aattctccta tatttttgat gaaggtttgt
      121 aataatgtaa gacatattga aatacaagtt gttggtgata tgtatggaaa tgtgtgttct
      181 ttaagtggtc gtgattgtac tacacaaaga agatttcaaa aaatttttga agaaggacca
      241 ccatctgttg taccatatcc tatatttcga gaaatggaaa aatcatctat acgattaact
      301 aaaatgatta aatatagagg tgctggaact attgaatatt tgtatgatca aataaataaa
      361 aaatattttt tcttagaatt aaatccaaga tta
//
```

Fig 24 y d page 8

RECTIFIED SHEET (RULE 91)
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```
LOCUS       AF137615       393 bp    DNA             INV       30-OCT-1999
DEFINITION  Plasmodium knowlesi acetyl-CoA carboxylase 1 (ACC1) gene, partial
            cds.
ACCESSION   AF137615
KEYWORDS    .
SOURCE      Plasmodium knowlesi.
  ORGANISM  Plasmodium knowlesi
            Eukaryota; Alveolata; Apicomplexa; Haemosporida; Plasmodium.
REFERENCE   1  (bases 1 to 393)
  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Growth of Toxoplasma gondii is inhibited by
            aryloxyphenoxypropionate herbicides targeting acetyl-CoA
            carboxylase
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 393)
  AUTHORS   Zuther,E., Johnson,J.J., Haselkorn,R., McLeod,R. and Gornicki,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-JUN-1999) Molecular Genetics and Cell Biology,
            University of Chicago, 920 East 58th Street, Chicago, IL 60637, USA
FEATURES             Location/Qualifiers
     source          1..393
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                     /db_xref="taxon:5850"
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                     /gene="ACC1"
                     /product="acetyl-CoA carboxylase 1"
     gene            <1..>393
                     /gene="ACC1"
     CDS             <1..>393
                     /gene="ACC1"
                     /codon_start=1
                     /product="acetyl-CoA carboxylase 1"
                     /translation="SQGGGGKGIRKVENEEEIKKAYTQVQMSLPNSPIFLMKVCSNVR
                     HIEIQVVGDMYGNVCSLSGRDCTTQRRFQKIFEEGPPSVVPPNIFREMEKASIRLTKM
                     IKYRGAGTIEYLYDQKKQTYYFLKLNPRL"
BASE COUNT      138 a     69 c     91 g     95 t
ORIGIN
        1 tcacaaggag gaggggggaa aggtattcgg aaagtggaga acgaagaaga aataaagaaa
       61 gcctacacac aagtgcaaat ggaattaccc aactcgccta tctttctaat gaaagtctgt
      121 agcaacgtta gacacatcga aatacaagtt gttgggggata tgtatggtaa tgtatgctcc
      181 cttagtggaa gagactgcac gacccaaagg aggttccaaa aaatttttga agaagggccc
      241 ccctcagttg tacctccgaa tatttttccgt gaaatggaaa aggcatccat acgtctaaca
      301 aaaatgataa aatatagagg tgcgggaact attgagtatt tatatgacca ggagaagcag
      361 acttattttt ttctcgaatt aaatcctcga ctg
//
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Fig 24d page 9

RECTIFIED SHEET (RULE 91)
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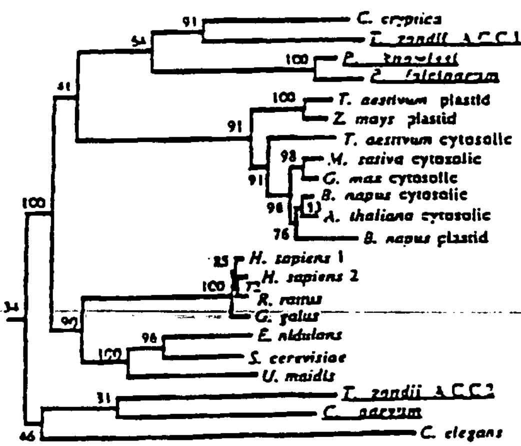

Fig 24 E

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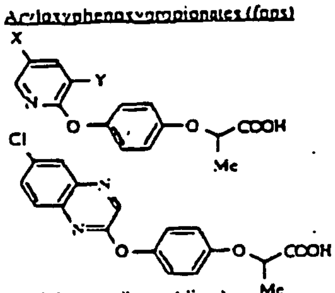
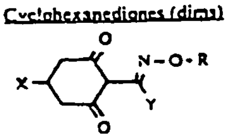
Aryloxyphenoxypropionates (fops)



Haloxyfop: X=CF_3. Y=Cl
Haloxyfop methyl ester
Haloxyfop ethyl ester
Fluazifop: X=CF_3. Y=H
Clodinafop: X=Cl. Y=F
Topik. clodinafop propargyl ester

Quizalofop
Targa. quizalofop ethyl ester
Agil. quizalofop 2-isopropylideneaminooxyethyl ester

Cyclohexanediones (dims)



Sethoxydim: X=$CH_2CH(CH_3)SCH_2CH_3$. Y=$CH_2CH_2CH_3$. R=CH_2CH_3
Cethoxydim: X=$C(SCH_3)CH_2$. Y=CH_2CH_3. R=$CH_2CH{=}CH_2Cl$
CH_2
Clethodim: X=$CH_2CH(CH_3)SCH_2CH_3$. Y=CH_2CH_3. R=$CH_2CH{=}CHCl$

Fig 24 F

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(71) Applicants: ARCH DEVELOPMENT CORPORATION [US/US]; University of Chicago, 5640 South Ellis Avenue, Chicago, IL 60637 (US). MRJ TRUST [US/US]; c/o Mark Rust, Barnes & Thronburg, 2600 Chase Plaza, 10 South LaSalle Street, Chicago, IL 60603 (US).

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(74) Agents: MARTIN, Alice, O. et al.; Barnes & Thornburg, 2600 Chase Plaza, 10 South LaSalle Street, Chicago, IL 60603 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *With international search report.*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/66154 A3

(54) Title: ANTI-MICROBIAL AGENTS, DIAGNOSTIC REAGENTS, AND VACCINES BASED ON UNIQUE APICOMPLEXAN PARASITE COMPONENTS

(57) Abstract: This invention relates uses of components of plant-like metabolic pathways not including psbA or PPi phosphorfructokinase and not generally operative in animals or encoded by the plastid DNA, to develop compositions that interfere with Apicomplexan growth and survival. Components of the pathways include enzymes, transit peptides and nucleotide sequences encoding the enzymes and peptides, or promoters of these nucleotide sequences to which antibodies, antisense molecules and other inhibitors are directed. Diagnostic and therapeutic reagents and vaccines are developed based on the components and their inhibitors. A cDNA sequence that encodes chorismate synthase expressed at an early state of Apicomplexan development, is disclosed and may be altered to produce a "knockout" organism useful in vaccine production.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/11478

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/002 G01N33/50 A61P33/02 //C12N15/60,C12N9/88, C12N1/11,A61K31/00,C07K16/40,G01N33/569,G01N33/577,C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ, MEDLINE, AIDSLINE, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 03661 A (ROBERTS FIONA ;ROBERTS CRAIG W (GB); ARCH DEV CORP (US); MCLEOD RI) 29 January 1998 (1998-01-29) page 6, line 15 -page 7, line 2 page 13, line 20 -page 15, line 10 page 22; table 1A page 29, line 11 - line 19 page 32, line 3 -page 34, line 19 page 37, line 17 -page 38, line 18 page 45, line 13 - line 19 page 69, line 16 -page 74, line 1 page 84, line 14 -page 88, line 2 page 92, line 2 -page 93, line 9 page 95, line 14 -page 97, line 3 page 106, line 20 -page 107, line 10 page 108, line 5 - line 18 page 125, line 18 -page 126, line 1 page 136, line 13 -page 137, line 3 claims 1-30 -/--	1-4,6,7

[X] Further documents are listed in the continuation of box C.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 2000	07/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stein, A

1

Inter onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	figures 1-14	5
A	EP 0 687 471 A (BAYER AG ;PARAVAX INC (US)) 20 December 1995 (1995-12-20) the whole document	7
A	ROBERTS FIONA ET AL: "Evidence for the shikimate pathway in apicomplexan parasites." NATURE (LONDON), vol. 393, no. 6687, 25 June 1998 (1998-06-25), pages 801-805, XP002153594 ISSN: 0028-0836 the whole document	1-7

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

ɔnal Application No
PCT/US 00/11478

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			EP	0918868 A	02-06-1999
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			JP	8188515 A	23-07-1996
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			SK	80195 A	08-05-1996